Supporting Information

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Enantioselective Aminohydroxylation of Styrenyl Olefins Catalyzed by an Engineered Hemoprotein

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I. Experimental Procedures

a. General.

Unless otherwise noted, all chemicals and reagents were obtained from suppliers Millipore Sigma and VWR and were used without further purification. NMR spectra were obtained using a Varian Innova 300 MHz, Bruker Prodigy 400 MHz, or Varian Innova 500 MHz instrument, using DMSO-d_6 as the solvent and are referenced to residual solvent signals. Data for 1H NMR are reported in the conventional form: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, hept = heptet, m = multiplet, br = broad), coupling constant (Hz), and integration. Sonication was performed using a Qsonica Q500 sonicator. Chemical reactions were monitored using thin layer chromatography (Merck 60 silica gel plates) and a UV lamp for visualization, if possible.

b. Chromatography.

Reverse-phase high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy (LC-MS) were carried out using Agilent 1200 series instruments and an Agilent XDB-C8 column (4.6 x 150 mm, 5 µm) with water and acetonitrile as the mobile phase. Both water and acetonitrile contained 0.1% acetic acid. Semi-preparative HPLC was performed using an Agilent XDB-C18 column (9.4 x 250 mm, 5 µm) with water and acetonitrile containing 0.1% acetic acid. Analytical chiral HPLC was performed using a Daicel Chiralpak IC column (4.6 x 250 mm, 5 µm) with hexanes and isopropanol as the mobile phase.

c. Cloning and site-saturation mutagenesis.

Plasmid pET22b(+) (Novagen) was used as a cloning and expression vector for all variants described in this paper. Site-saturation mutagenesis was performed using modified QuikChange™ mutagenesis protocol using 22-codon trick.[1] The PCR products were further digested with *Dpn*I, purified with New England Biolabs gel purification kit, and the gaps were repaired using Gibson Mix™.[2] Without further purification, 1 µL of the ligation mixture was used to transform 50 µL of electrocompetent *E. coli* strain BL21 E. cloni (Lucigen).

d. Cloning for cytochrome *c*.

The gene encoding *Rhodothermus marinus* cytochrome *c* (UNIPROT ID B3FQS5) was originally obtained as a single gBlock (IDT), codon-optimized for *E. coli*, and cloned using Gibson assembly into pET22b(+) between restriction sites *Nde*I and *Xho*I in frame with an N-terminal pelB leader sequence and a C-terminal His-tag. This plasmid was used as the basis of all *Rma* cytochrome *c*

variants described in this study. All plasmids and ligation mixtures containing the gene encoding a cytochrome *c* variant were co-transformed with the cytochrome *c* maturation plasmid pEC86[3] encoding the gene ccmABCDEFG for maturation into BL21 E. cloni (Lucigen).

Cytochrome *c* site-saturation mutagenesis was performed using a further modified protocol with 22-codon trick. Instead of one long-range PCR of the entire plasmid, two PCRs were performed, pairing the forward site-saturation primer mixture with a pET22b(+) internal reverse primer, and the reverse site-saturation primer mixture with a pET22b(-) internal forward primer. The two PCR products were purified together using New England Biolabs gel purification kit, and the gaps were repaired using Gibson Mix. The ligation mixture was then used directly for co-transformation with the pEC86 plasmid into BL21 E. cloni electrocompetent cells.

e. Expression of $P411_{BM3}$, globin, and cytochrome *c* variants.

Escherichia coli BL21 E. cloni electrocompetent cells were transformed with pET22b(+) constructs encoding various $P411_{BM3}$ and protoglobin variants. The cells were grown overnight in 6-mL Luria-Bertani (LB_{amp}) medium, supplemented with ampicillin, and 5 mL of this preculture were used to inoculate 45 mL of Hyperbroth (HB_{amp}) medium. The expression culture was incubated at 37 ℃ and 250 rpm for two hours. Then, the expression culture was cooled on ice for 20 minutes and was induced with 1 mM 5-aminolevulinic acid (ALA) and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), final concentrations. Cells were allowed to express at 22 ℃ and 130 rpm for 24 hours. Once expression was finished, the cultures were centrifuged (4,000 g, 5 minutes, and 4 ℃) and the pellets were resuspended to an OD_{600} of 30 in M9-N minimal medium at pH 7.4.

To express cytochrome *c* variants, *E. coli* BL21 E. cloni electrocompetent cells were transformed with pET22b(+) constructs encoding various cytochrome c variants, along with the pEC86 construct for cytochrome c maturation. The cells were grown overnight in 5 mL Luria-Bertani ($LB_{amp+chl}$) medium, supplemented with ampicillin and chloramphenicol, and 0.5 mL of this preculture were used to inoculate 30 mL of Hyperbroth ($HB_{amp+chl}$) medium. The expression culture was incubated at 37 ℃ and 250 rpm until an OD600 of 0.5 – 0.7 was reached. Then, the expression culture was cooled on ice for 25 minutes and was induced with 200 µM ALA and 20 µM IPTG, final concentrations. Cells were allowed to express at 22 ℃ and 250 rpm for 20 – 24 hours. Once expression was finished, the cultures were centrifuged (4,000 g, 5 minutes, 4 ℃) and the pellets were resuspended to an OD_{600} of 30 in M9-N minimal medium at pH 7.4.

f. Purification of cytochrome *c* variants.

Harvested cells from 300 – 600 mL of expression cultures were resuspended in buffer containing 200 mM potassium phosphate, 100 mM NaCl, and 20 mM imidazole (pH is adjusted to 7.5 at 25 ℃). Cells were lysed by sonication (4 minutes, 1 seconds on, 1 seconds off, 40% intensity; Qsonica Q500 sonicator). The sonication was repeated once more, and then the partially lysed cells were placed in a 55 ℃ heat bath for 20 minutes and then moved to 75 ℃ heat bath for an additional 10 minutes. The cell debris was removed by two serial centrifugation steps (5,500 g, 20 minutes, 4 ℃ and then 30,000 g, 30 minutes, 4 ℃). The supernatant was filtered through a 0.2-µm cellulose acetate filter and purified using a 1-mL Ni-NTA column using AKTA purifier FPLC system by running a gradient from 20 mM to 500 mM imidazole over 10 column volumes. Pure fractions were determined using SDS-PAGE and were pooled and concentrated using a 3-kDA molecular weight cut-off centrifugal filter. The concentrated fractions were washed twice with 0.1 M phosphate buffer at pH 7.5 and were dialyzed overnight into 0.05 mM phosphate buffer at pH 7.5 using a 3-kDA molecular weight cut-off dialysis tubing. The dialyzed protein was frozen on dry ice and stored at -20 ℃.

g. Reaction setup and product characterization.

In preparation for the reaction, a 250-mM glucose solution was prepared in M9-N medium (pH 7.4), filtered through a 0.2-µm cellulose acetate filter, and placed in the anaerobic chamber for at least a day. The cells were degassed by sparging with argon for 30 minutes at 4 ℃. To 2-mL screw cap vials were added 20 µL of a stock solution containing 1,000 U/mL glucose oxidase and 14,000 U/mL catalase prepared in double-distilled water. Resuspended cells were added to the screw cap vials, and all vials containing cells or reagent were immediately transferred into an anaerobic chamber with oxygen concentration below 15 ppm. In the chamber, 40 µL of the glucose solution and the reagents (typically 200 mM-1 M stocks in DMSO) were added. The final reaction volume was 400 µL for all reactions on analytical scale. The reaction mixture was typically shaken at room temperature for 24 hour at 600 rpm. After completion of the reaction, acetonitrile was added to quench the reaction and precipitate protein. The mixture was frozen for 4 hours at -20 ℃ and then defrosted at room temperature for an hour to encourage cell lysis. The defrosted mixture was transferred to a 1.7-mL microcentrifuge tube and was centrifuged at 14,000 g for 5 minutes. To determine the yield, 200 µL of the supernatant were transferred to an HPLC vial with insert and analyzed by reverse-phase HPLC-MS. Protein concentration in the cell was determined by performing hemochrome assay on the cell lysate. Lysate was obtained by sonication (4 minutes, 1 seconds on, 1 seconds off, 40% intensity) followed by centrifugation (4,500 g, 10 minutes, 4 ℃) to remove cell debris.

In order to determine the enantioselectivity, acetonitrile was removed from the remaining supernatant, and the solution was made more basic by adding 80 μL of 1 M NaOH (aq). For derivatization, 10 – 15 μL of 10% benzoyl chloride solution in acetonitrile were added, and the solution was allowed to shake at room temperature (25 ℃) and 600 rpm for 4 hours. Derivatized product was extracted with ethyl acetate and analyzed by chiral normal-phase HPLC to determine the enantiomeric excess (ee).

h. Reaction screening in 96-well plate format.

After a single-site-saturation library was generated, 88 single colonies were randomly picked and cultured in 300 μL of LB medium with 0.1 mg/mL ampicillin (LB_{amp}) in a sterilized 96-well culture plate. The plate contained four wells inoculated with parent single colonies, and four sterile wells. The cells in LB medium were cultured at 37℃, 230 rpm, and 80% relative humidity for 10-12 hours. A separate sterilized 96-well culture plate was filled with 1000 μL of Hyperbroth medium containing 0.1 mg/mL ampicillin (HB_{amp}) in each well. The new plate with HB_{amp} was inoculated with the LB preculture (50 μL/well) and incubated at 37℃, 230 rpm, and 80% relative humidity for 3 hours. The plate was cooled on ice for 30 minutes, induced with 20 μM IPTG and 200 μM 5-aminolevulinic acid (final concentrations), and then expression was conducted at 22 ℃ and 230 rpm for 20 hours. The cells were pelleted (4,500 g, 5 minutes), an oxygen depletion system[4] (20 μL/well of a stock solution containing 14,000 U/mL catalase from *Corynebacterium glutamicum* and 1,000 U/mL glucose oxidase from *Aspergillus niger* in M9-N medium) was added, and the mixture was gently resuspended. The 96-well plate was transferred to an anaerobic chamber, where argon-sparged 50 mM D-glucose in 0.1 M potassium phosphate buffer (pH 8.0) was added (300 μL/well). The nitrene source (10 μL/well, 400 mM in DMSO) and the alkene (10 μL/well, 400 mM in DMSO) were subsequently added, and the plate was sealed with an aluminum foil and shaken at 600 rpm at room temperature for 20-24 hours.

Once the plate was taken out of the anaerobic chamber and the seal was removed, acetonitrile (800 μL/well) and internal standard (10 μL/well of a 200mM DMSO stock) were added. The wells were mixed by pipetting and the plate was left to shake at 600 rpm at room temperature for an hour. The plate was then centrifuged (5,000 g, 5 minutes), and the supernatant (200 μL/well) was filtered through an AcroPrep 96-well filter plate (0.2 μm) into a shallow 96-well plate for reverse-phase HPLC-MS analysis.

i. Protein lysate preparation.

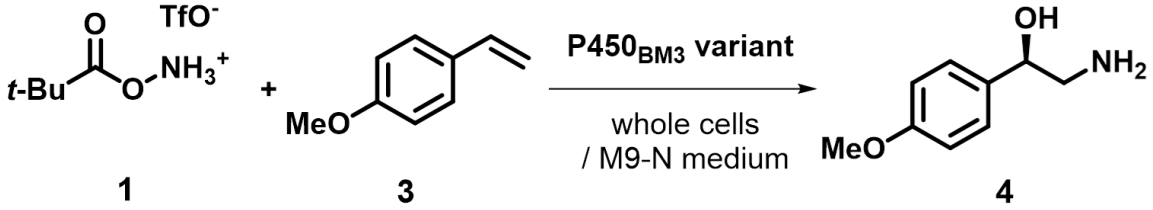
Protein-containing lysate was prepared from pelleted and resuspended cells adjusted to a given OD_{600}. Protein lysates for P450s were obtained by sonication (1 second on, 1 second off, 3 minutes with 30% - 40% amplitude, on wet ice) in the presence of BugBuster™ reagent. The sonicated cell mixture was distributed into 2.0-mL Eppendorf tubes and then centrifuged (14,000 g, 10 minutes, 4 ℃) to remove cell debris. The supernatant was immediately transferred to a different container, filtered through a 0.45 μm cellulose filter, and used to determine protein concentration. Protein lysates for cytochrome *c* variants were obtained by freezing at -20 ℃ for 3 hours, then thawing and performing a heat treatment (55 ℃, 25 minutes). The heat-treated mixture was centrifuged (5,000 g, 10 minutes, 4 ℃) to remove cell debris. The supernatant was filtered through a 0.2 μm cellulose filter.

j. Determining protein concentration.

All hemoprotein concentrations were determined in triplicate using the hemochrome assay. A solution of 1 M NaOH (0.4 mL) was mixed with pyridine (1 mL) in a 1.7 mL-Eppendorf tube. The tube was centrifuged (14,000 g, 1 minutes) to separate the excess aqueous layer and give a pyridine-NaOH solution on top. Separately, a solution of sodium dithionite (10 mg/mL) was prepared in a potassium phosphate buffer (pH 8.0). To a cuvette containing 800 μL protein solution in potassium phosphate buffer (pH 8.0), 50 μL of dithionite solution and 150 μL pyridine-NaOH solution were added. The solution was mixed, the cuvette was sealed with Parafilm, and the UV-Vis spectrum of the reduced hemoprotein was recorded immediately. Hemoprotein concentrations were determined using $\varepsilon_{550\text{-}535} = 22.1\ mM^{-1}cm^{-1}$ for heme *c*.[5] This value was also compared with an alternative method of hemochrome assay where potassium ferricyanide solution is used,[6] and the agreement was within 3%.

II. Supplementary Figures and Tables

Supplementary Table 1. Aminohydroxylation of 4-vinylanisole (**3**) with variants of cytochrome $P450_{BM3}$. [a]



Variant	Mutations relative to the wild-type $P450_{BM3}$	Yield [%]
pET22b(+) vector	N/A: not expressing any hemoprotein	0.9
$P450_{BM3}$	none	0.8
$P411_{BM3}$	C400S	4.1[b]
$P411_{BM3}$ T268A	T268A, C400S	2.4
$P411_{BM3}$ F87A T268A [c]	F87A, T268A, C400S	1.5
$P411_{BM3}$ F87V T268A	F87V, T268A, C400S	1.2
$P411_{BM3}$ CIS T438S ("P")	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, T438S, E442K	1.9
$P411_{BM3}$ CIS T438S A268T	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, A290V, L353V, I366V, C400S, T438S, E442K	1.7
"P-I263F" [c]	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, I263F, T268A, A290V, L353V, I366V, C400S, T438S, E442K	2.4
"P-I263F" F263M	P-I263F I263M	1.3
"P-I263F" F263Y	P-I263F I263Y	1.2
"P-I263F" A328V	P-I263F A328V	3.4[b]
"P-I263F" V87A A328V	P-I263F F87A A328V	1.4
"A10" [d]	P-I263F A328V, L437V	1.9
"A10" C400H	P-I263F A328V, C400H, L437V	1.2
"A10" C400A	P-I263F A328V, C400A, L437V	1.2
"A10" C400	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, I263F, T268A, A290V, A328V, L353V, I366V, L437V, T438S, E442K	1.0
P-I263F V87A A328V A268G A82I ("A82I")	P-I263F A82I, F87A, T268G, A328V	1.1
P-I263F V87A A328V	P-I263F A82L, F87A, T268G, A328V	1.2

A268G A82L (“A82L”)		
"A82L" A78V F263L E267D	P-I263F A78V, A82L, F87A, F263L, E267D, T268G, A328V	1.7
“A82L” A78V F263L	P-I263F A78V, A82L, F87A, F263L, T268G, A328V	1.1
$P411_{BM3}$ H2-4-D4	L75A, V78A, F87V, P142S, T175I, M177A, L181A, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, L437A, E442K	4.4[(b)]
$P411_{BM3}$ H2-A-10	L75A, V78A, F87V, P142S, T175I, L181A, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, E442K	3.8[(b)]
$P411_{BM3}$ H2-5-F10	L75A, V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, I263A, T268A, A290V, L353V, I366V, C400S, L437A, E442K	1.5
$P411_{BM3}$ CIS I263W C400H	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, I263W, T268A, A290V, L353V, I366V, C400H, E442K	1.1
$P411_{BM3}$ CIS L437F T438Q	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, L437F, T438Q, E442K	1.4
$P411_{BM3}$ CIS L437F T438Q L75Y	L75Y, V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, L437F, T438Q, E442K	1.3
$P411_{BM3}$ CIS L437F T438Q L181V	V78A, F87V, P142S, T175I, L181V, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, L437F, T438Q, E442K	1.5
$P411_{BM3}$ CIS L437F T438Q L75Y L181I	L75Y, V78A, F87V, P142S, T175I, L181I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, L437F, T438Q, E442K	1.3
$P411_{BM3}$ CIS I263G L437F	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, I263G, T268A, A290V, L353V, I366V, C400S, L437F, E442K	1.4
$P411_{BM3}$ CIS I263G L437F V87L	V78A, F87L, P142S, T175I, A184V, S226R, H236Q, E252G, I263G, T268A, A290V, L353V, I366V, C400S, L437F, E442K	2.0
$P411_{BM3}$ CIS I263G L437F V87L L181R	V78A, F87L, P142S, T175I, L181R, A184V, S226R, H236Q, E252G, I263G, T268A, A290V, L353V, I366V, C400S, L437F, E442K	2.1
$P411_{BM3}$ CIS V87T T438C	V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, T438C, E442K	1.2
$P411_{BM3}$ CIS V87T I263G L437F T438C Q674STOP	V78A, F87T, P142S, T175I, A184V, S226R, H236Q, E252G, I263G, T268A, A290V, L353V, I366V, C400S, L437F, T438C, E442K, Q674STOP	1.6

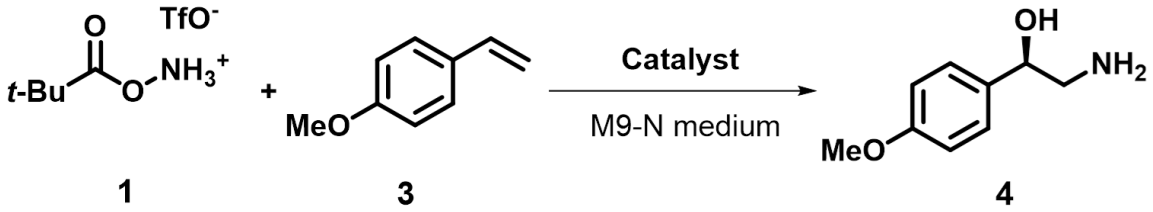
(a) Reactions were performed using whole cells at OD_{600} = 30 with 5 mM of each substrate; results are the average of duplicate reactions. N.D. = none detected.

(b) Enantiomeric excess was measured for $P450_{BM3}$ variants that gave highest yields. The ee ranged from 0 to 11%, with $P411_{BM3}$ giving 11% ee.

(c) Variants identified for regioselective intramolecular C-H amination.[7]

(d) Variant identified for aziridination of styrenes.[8]

Supplementary Table 2. Aminohydroxylation of 4-vinylanisole with hemin and various hemoproteins. [(a)]



1 3 4

Catalyst	Note	Yield [%]
Whole cells expressing pET22b(+) pEC86 vector	pEC86 encodes ccmABCDEFG to aid the maturation of cytochrome *c* proteins	0.8
Hemin [(b)]	Concentration: 10 µM	0.1
Hemin + imidazole [(b)]	10 µM hemin + 1 mM imidazole	0.08
Hemin + bovine serum albumin [(b)]	Concentration: 10 µM each	0.1
Bovine serum albumin only	Concentration: 10 µM	N. D.
Myoglobin	From equine heart, concentration: 10 µM	2.2
Rhodothermus marinus (*Rma*) cytochrome *c*		4.0 [(c)]
Hydrogenobacter thermophilus cytochrome *c*		1.1
Rhodopila globiformis cytochrome *c*		1.0
Rma cytochrome *c* M100D		2.9
Rma cytochrome *c* "TDE" [(d)]	Mutations from the wildtype: V75T M100D M103E	3.8

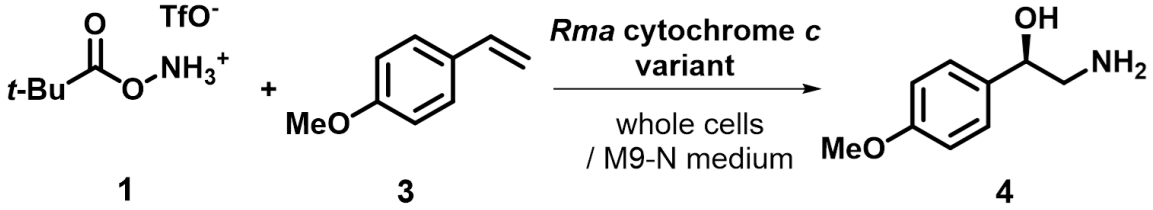
(a) Reactions were performed with 5 mM of each substrate; results are the average of triplicate reactions. N.D. = none detected. Whole cells resuspended in M9-N medium to OD_{600} = 30 were generally used, unless otherwise specified.

(b) Hemin and other additives were dissolved in M9-N medium without any whole cells to specified final concentrations. Hemin was reduced using sodium dithionite solution (10 mg/mL) prior to reaction.

(c) Enantiomeric excess was measured for the product of wild-type *Rma* cytochrome *c* reaction. It was determined to be 32 % ee.

(d) Variant identified for carbene Si-H insertion.[9]

Supplementary Table 3. Aminohydroxylation data presented in Figure 2A of the main text.



Variant	Mutations from the wild-type *Rma* cytochrome c	TTN	Yield [%]	ee [%]
Rma cytochrome *c*	None	40	4.8	32
"M100S"	M100S	56	5.3	39
"SG"	M100S, M103G	74	7.2	60
"SPG"	M100S, T101P, M103G	86	7.3	51
"VSPG"	M99V, M100S, T101P, M103G	360	15.0	73
VSPG T98L	T98L, M99V, M100S, T101P, M103G	640	25.8	76
VSPG M76Q T98L	M76Q, T98L, M99V, M100S, T101P, M103G	1800	76.1	85
"TQL"	Y44T, M76Q, T98L, M99V, M100S, T101P, M103G	2500	90.4	90

Reactions were performed using whole cells at OD_{600} = 30 with 10 mM of each substrate; results are the average of triplicate reactions. Site-saturation libraries were generated and tested in the 96-well expression and reaction format discussed in the general procedures. Beneficial mutations were identified, and the best variants were grown in 30 mL expression cultures and re-tested. The TTN and ee reported in this table are obtained with the cultures grown at 30-mL scale.

Supplementary Table 4. Summary of directed evolution for aminohydroxylation.

Generation	Parents	Saturated sites	Mutation identified
1	Wildtype *Rma* cyt *c*	M100X	M100S, M100H
2	M100S, M100H	M103X	M100S M103G ("SG")
3a	"SG"	V75X, L105X	None
3b	"SG"	T101X	T101A, T101P
4a	"SG" T101A	M99X	T101P M99V ("VSPG")
4b	"SG" T101P		
5	"VSPG"	T98X	T98L
6	"VSPG" T98L	M76X, Y44X	M76Q
7	"VSPG" M76Q T98L	Y44X	Y44T

Reactions were performed in the 96-well expression and reaction format, with 10 mM of each screening substrates. Active site residues T98, M99, M100, T101, M103, and L105 are located in the loop distal to the heme cofactor of *Rma* cytochrome *c*. Active site residues V75 and M76 are located on the helix on the distal face of heme. Y44 is near the region where the active site is exposed to the aqueous environment.

Supplementary Table 5. Description of the substrate scope presented in Scheme 1 of the manuscript.

Product	OD_{600}	Reactant concentration	Temperature	TTN	Yield [%]	ee [%]
4	30	10 mM each	RT	2500	90.4	90
5	30	10 mM each	RT	470	20.1	63
6	60	10 mM each	7 °C [(a)]	1900	96.5	80
7	60	10 mM each	RT	180 [(c)]	12.2	67
8	30	10 mM each	RT	1700	60.3	53
9	30	10 mM olefin substrate, 25 mM nitrene precursor **1**	RT	1200	50.8	92
10	30	10 mM olefin substrate, 20 mM nitrene precursor **1**	RT	190 [(c)]	8.5	88
11	30	10 mM olefin substrate, 20 mM nitrene precursor **1**	RT	330	13	92
12	30	10 mM olefin substrate, 20 mM nitrene precursor **1**	37 °C	150 [(c)]	7.3	82
13	60	20 mM each	37 °C	80 [(c)]	8.6	80
14	30	10 mM olefin substrate, 30 mM nitrene precursor **1**	20 °C	2400	77.2	85
15	30	10 mM each	RT	750	27.8	45
16	30	10 mM each	RT	2400 [(b)]	76.8	93 [(d)]

Room temperature (RT) was typically 23 – 25 °C. Reactions performed at specified temperatures other than RT were shaken in a temperature-controlled shaker. Nitrene precursor **1** was used for all reactions.

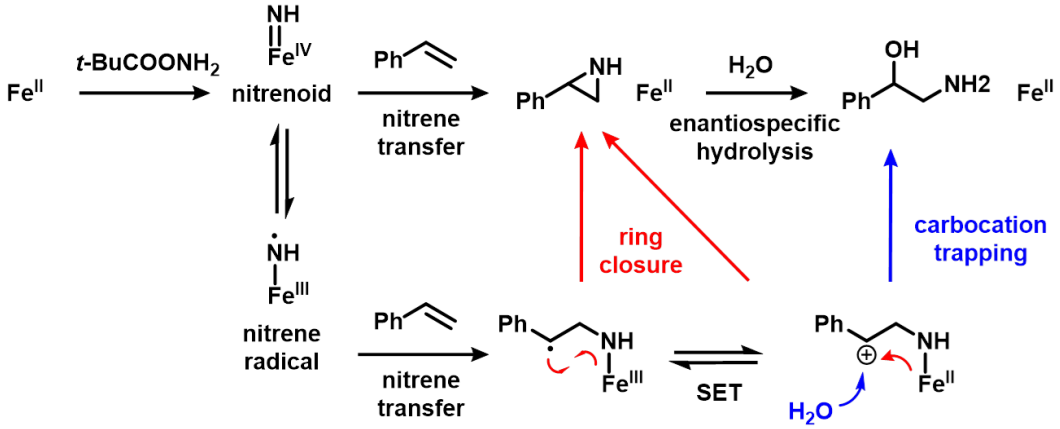
(a) Olefin reagents were light or temperature sensitive, and often generate white precipitate during room temperature reactions.

(b) For this substrate, TTN was calculated as the concentration of all aminohydroxylation products over the concentration of protein.

(c) Cells were allowed to express protein at 25 °C, yielding higher concentration of protein for a given OD_{600}.

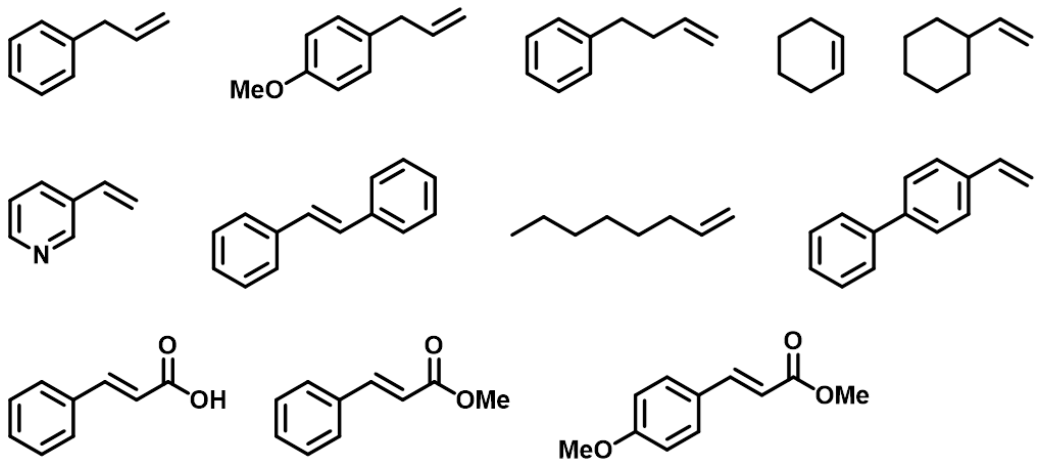
(d) The product has diastereomeric ratio of 87:13 for (*1R,2S*)-(*1S,2R*)-**15** and (*1R,2R*)-(*1S,2S*)-**15**, respectively. The ee refers to the major diastereomer only.

Supplementary Figure 1. Mechanistic hypothesis for styrenyl olefin hydroxylation.



Two processes for olefin aminohydroxylation are possible, involving either a nitrene or nitrene radical intermediate. Nitrene transfer to styrene could give an aziridine, which can hydrolyze to the amino alcohol product. Alternatively, a nitrene radical intermediate may give rise to carbon-centered radical or a carbocation intermediate, which may either rearrange to the aziridine or proceed to the amino alcohol product without the intermediacy of an aziridine species.

Supplementary Figure 2. Substrates showing low reactivity (<30 TTN) toward aminohydroxylation by the cytochrome *c* TQL variant.



III. Synthesis of Substrates and Product Standards.

Most chemicals were purchased from commercial vendors and were used without further purification. All olefin substrates and product standards were purchased from commercial suppliers (Fisher Scientific, Enamine, Millipore Sigma, VWR, TCI America). Nitrene precursors were synthesized. All nitrene precursors are known compounds, and their spectral data are in agreement with reported values.

a. Nitrene precursor 1 (*O*-pivaloylhydroxyamine triflic acid).

Pivalic anhydride (18.3 mL, 90.1 mmol) was added to a solution of *N*-Boc hydroxylamine (10.0 g, 75.1 mmol) in chloroform (200 mL). The reaction mixture was refluxed for 16 hours. The mixture was then quenched with saturated sodium bicarbonate solution and diluted with dichloromethane. The organic phase was washed three times with saturated sodium bicarbonate solution. The organic phase was then dried with magnesium sulfate, filtered, and evaporated under reduced pressure. The white solid obtained was dissolved in anhydrous diethylether (200 mL) and cooled on an ice water bath. Triflic acid (7.2 mL, 81.2 mmol) was added dropwise. The reaction was then allowed to reach room temperature and was diluted with hexanes to precipitate the product. The product was obtained by filtration, and excess solvent was removed under reduced pressure. The product is a white solid (10.5 g, 52% over 2 steps). The NMR spectrum agreed with the spectrum previously reported.[10]

^{1}H NMR (400 MHz, DMSO-d_6): δ 1.20 (s, 9H).

b. Nitrene precursor 17 (*O*-(*tert*-butoxycarbonyl)hydroxylamine).

Hydroxylamine hydrochloride (1.94 g, 28 mmol) in 50 mL 50% (v/v) dioxane was adjusted to pH 10 with 4 N sodium hydroxide, on ice water bath. Di-tert-butyl dicarbonate (15.7 g, 72 mmol) was added and the pH was maintained at 10 by additional adjusting with sodium hydroxide. After 30 minutes, the reaction was allowed to reach room temperature, and a small amount of distilled water was added to dissolve precipitate. After 90 minutes, 3 N hydrochloric acid was added to acidify the solution to pH 2, and the product was extracted three times with 50 mL chloroform. The chloroform solution was washed with brine, dried over magnesium sulfate, and evaporated under reduced pressure. Tert-butanol and excess solvent were further evaporated by freeze-pump-thaw under reduced pressure. The product was a clear liquid at room temperature (2.66 g, 20 mmol). The NMR spectrum agreed with the spectrum of **16** reported.[11]

^{1}H NMR (400 MHz, DMSO-d_6): δ 1.48 (s, 9H).

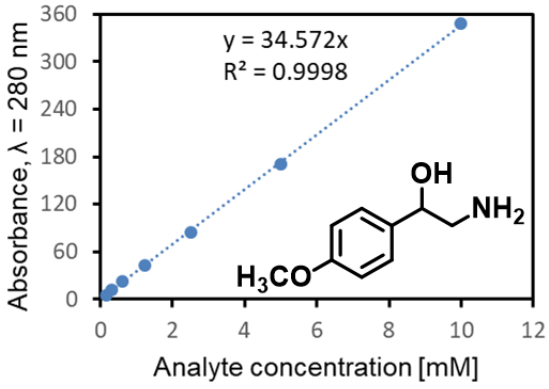
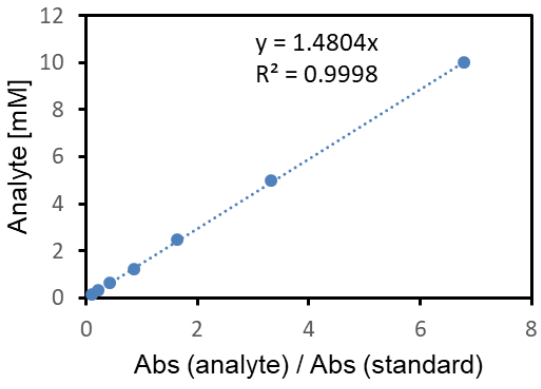
c. Nitrene precursor 18 (*O*-pivaloylhydroxyamine trifluoroacetic acid).

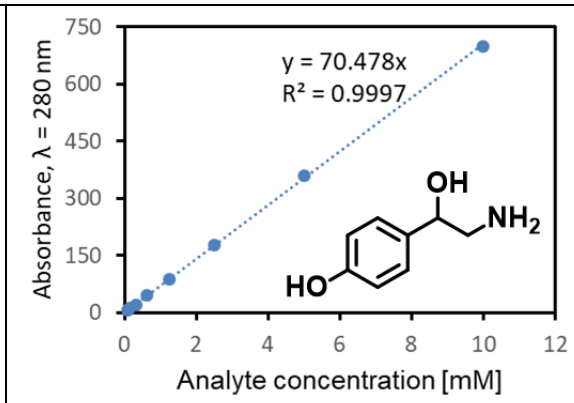
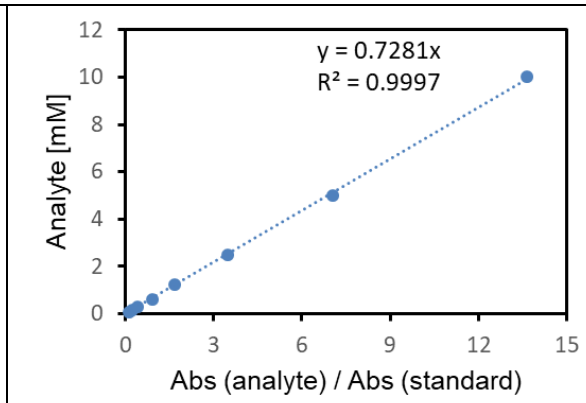
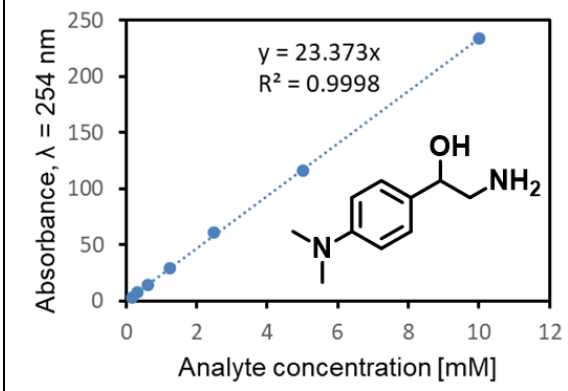
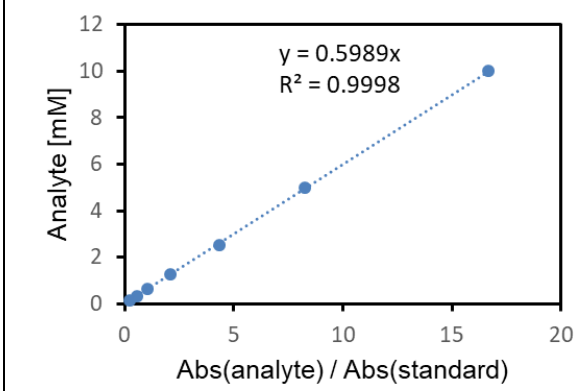
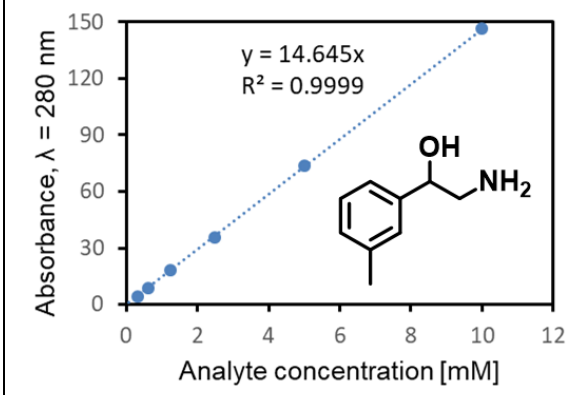
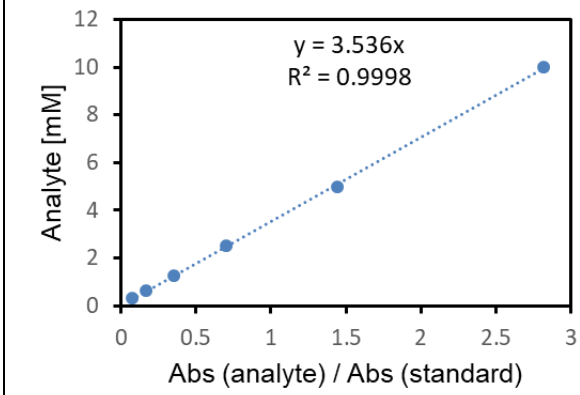
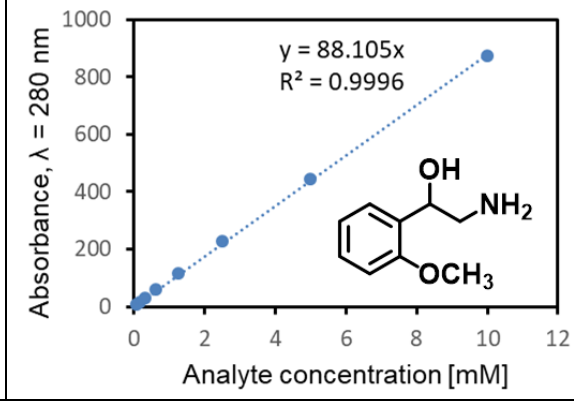
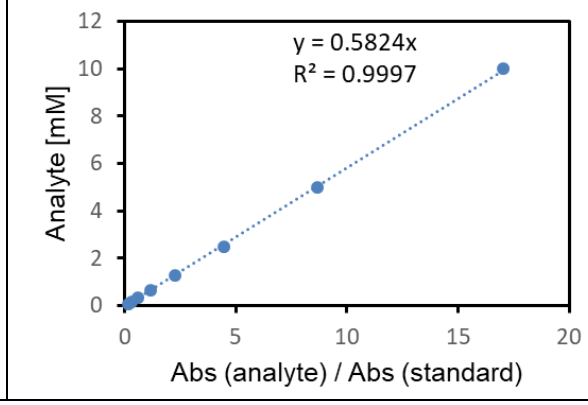
Pivalic anhydride (3.66 mL, 18.0 mmol) was added to a solution of *N*-Boc hydroxylamine (2.00 g, 15.0 mmol) in chloroform (40 mL). The reaction mixture was refluxed for 16 hours. The mixture was then quenched with saturated sodium bicarbonate solution and diluted with dichloromethane. The organic phase was washed three times with saturated sodium bicarbonate solution. The organic phase was then dried with magnesium sulfate, filtered, and evaporated under reduced pressure. The white solid obtained was dissolved in anhydrous diethylether (40 mL). Trifluoroacetic acid (1.4 mL) was added dropwise at 0 °C. The reaction was allowed to reach room temperature and was diluted with hexanes to precipitate the product. The reaction mixture was filtered to obtain the desired product as a white solid (1.40 g, 35%). The NMR spectrum agreed with the spectrum for nitrene precursor **1**.

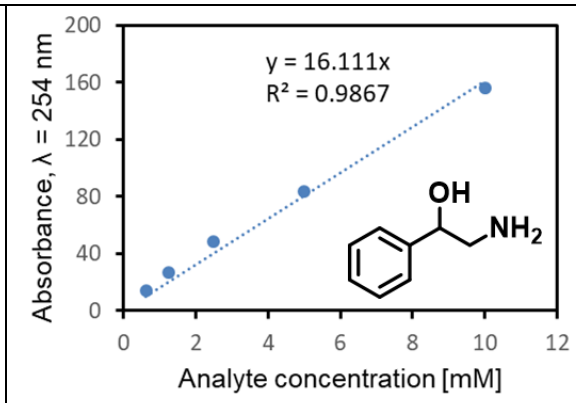
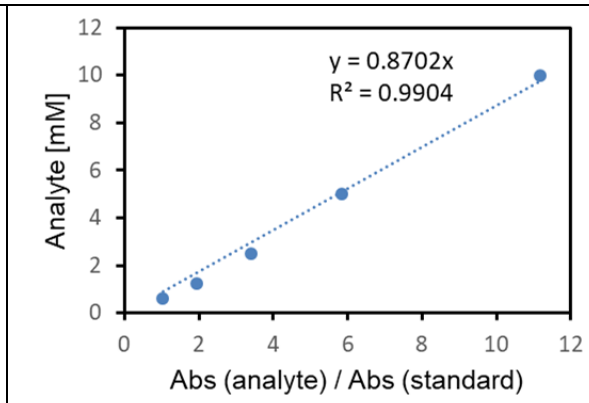
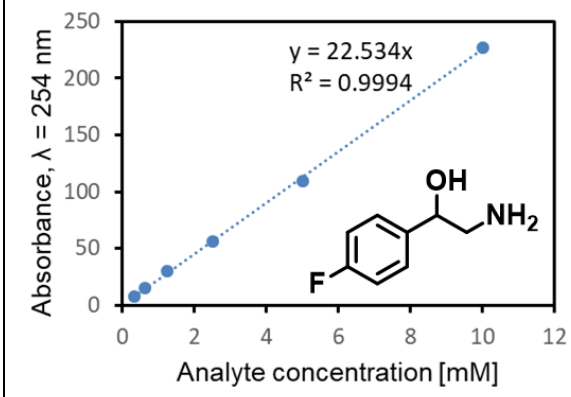
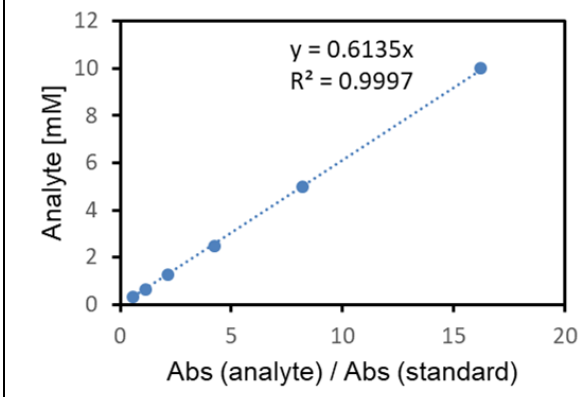
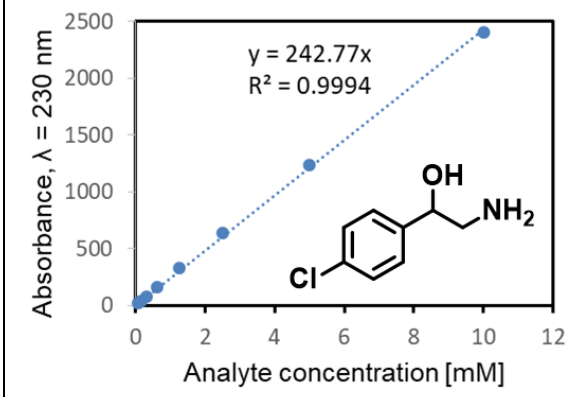
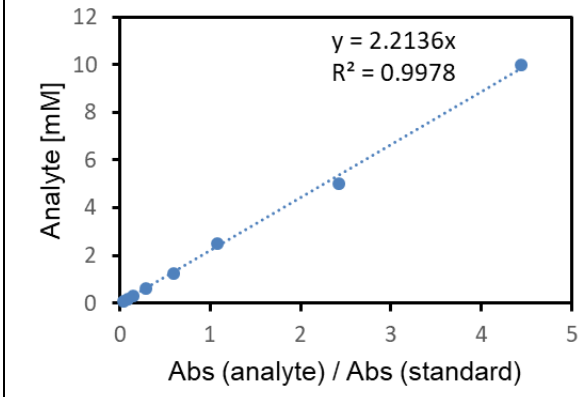
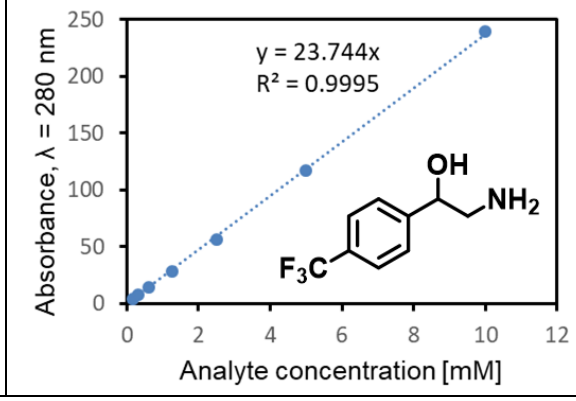
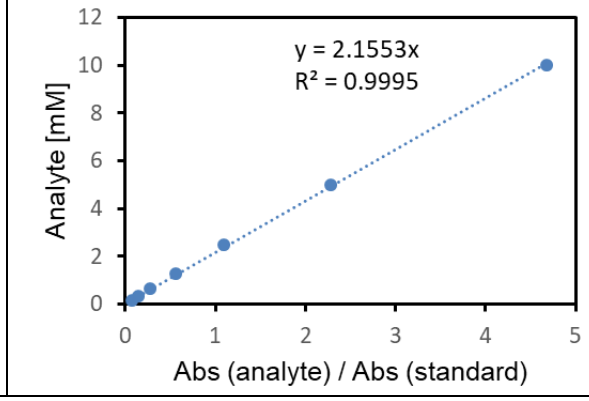
IV. Characterization of Products and HPLC Calibration Curves

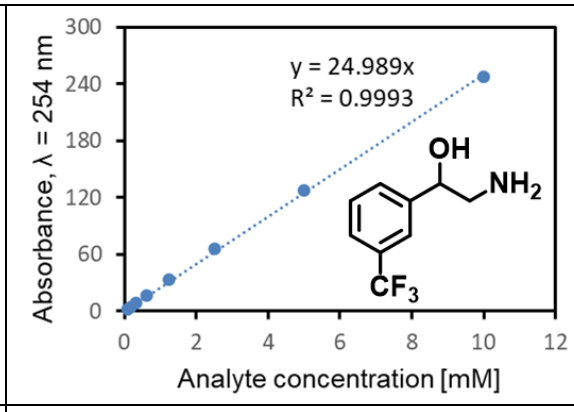
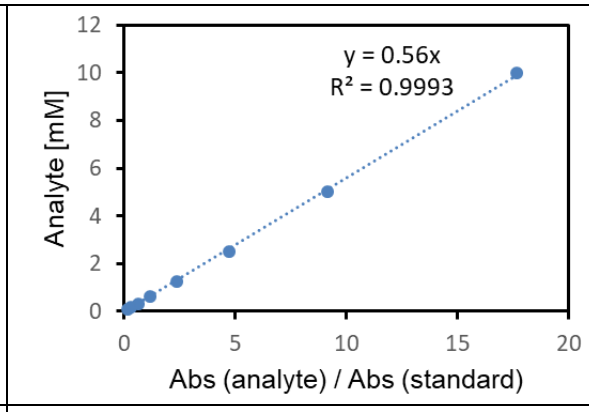
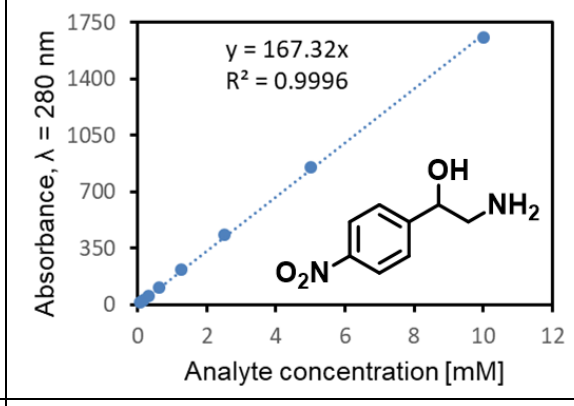
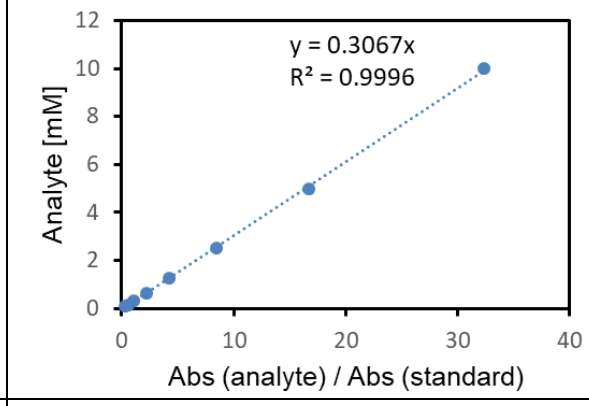
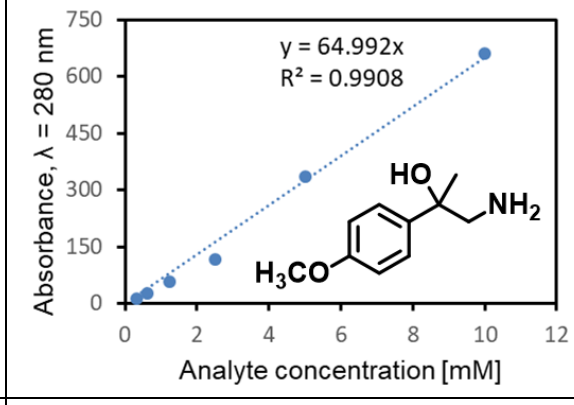
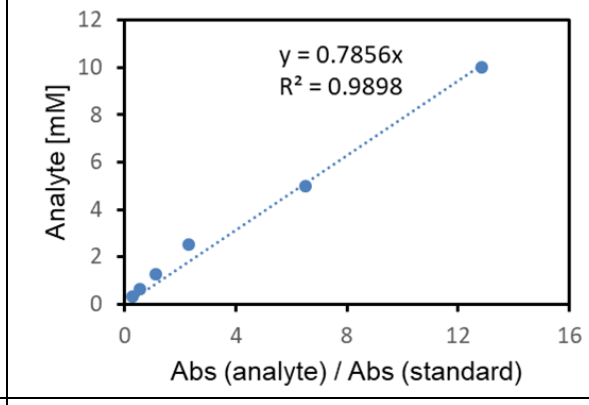
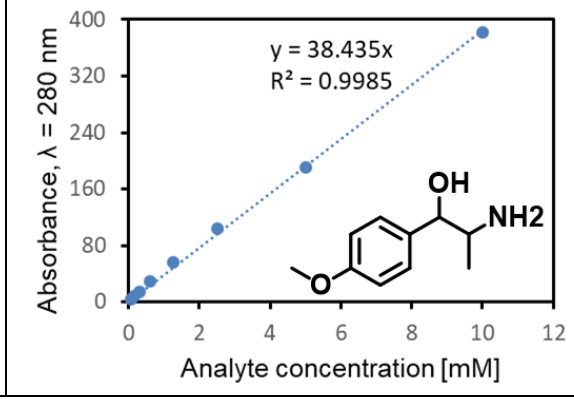
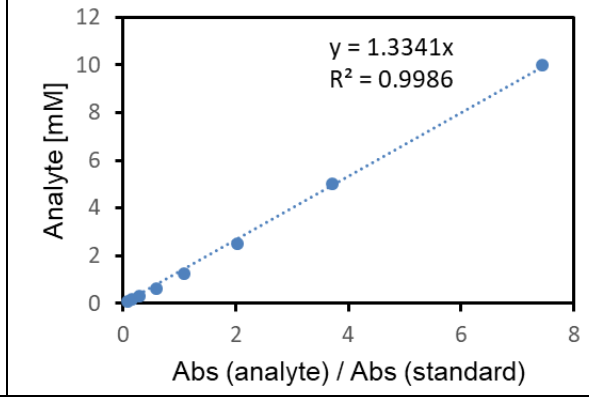
UV absorbance of the product standards was plotted as a function of the product concentration. Data were fitted with a linear trendline with zero intercept to show the linearity of UV absorption. Calibration curves with an internal standard were created for the determination of yield and TTN. The concentration of analytes was plotted as a function of the UV absorbance ratio of the analyte over an appropriate internal standard. Product standards analyzed based on absorbance at 254 nm or shorter wavelengths were calibrated with 1,3,5-trimethoxybenzene (1.25 mM, final concentration) as the internal standard. Product standards analyzed based on absorbance at 280 nm were calibrated with acetophenone (1.25 mM, final concentration) as the internal standard. The identity of the products was confirmed by HPLC-MS co-injections of reaction mixtures with authentic product standards.

The appropriate wavelength for each compound was determined based on their UV absorption maxima, retention time using the reverse-phase HPLC method, and the presence of cell impurities sharing the same retention time. Methods were adjusted so that none of the products elute at the same time with any of the cell impurities. For all analysis, water and acetonitrile containing 0.1% acetic acid were used as eluents. The method used 5 – 40% acetonitrile (0 – 3 minutes), 40 – 95% acetonitrile (3 – 4 minutes), 95% acetonitrile (4 – 5 minutes), followed by one minute of postrun (5% acetonitrile). The flow rate was 1.5 mL/minute, and the column was maintained at 37 °C for the entire method.

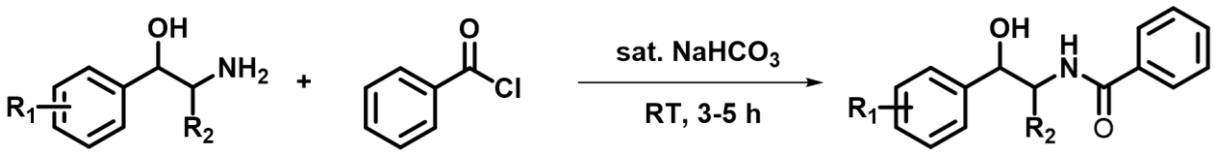
Product	Absorbance vs. concentration	Calibration with internal standard
4		

5	 y = 70.478x R² = 0.9997 Absorbance, λ = 280 nm Analyte concentration [mM] OH NH2 HO	 y = 0.7281x R² = 0.9997 Analyte [mM] Abs (analyte) / Abs (standard)
6	 y = 23.373x R² = 0.9998 Absorbance, λ = 254 nm Analyte concentration [mM] OH NH2 N	 y = 0.5989x R² = 0.9998 Analyte [mM] Abs(analyte) / Abs(standard)
7	 y = 14.645x R² = 0.9999 Absorbance, λ = 280 nm Analyte concentration [mM] OH NH2	 y = 3.536x R² = 0.9998 Analyte [mM] Abs (analyte) / Abs (standard)
8	 y = 88.105x R² = 0.9996 Absorbance, λ = 280 nm Analyte concentration [mM] OH NH2 OCH3	 y = 0.5824x R² = 0.9997 Analyte [mM] Abs (analyte) / Abs (standard)

9	 y = 16.111x R² = 0.9867 Absorbance, λ = 254 nm Analyte concentration [mM] OH NH2	 y = 0.8702x R² = 0.9904 Analyte [mM] Abs (analyte) / Abs (standard)
10	 y = 22.534x R² = 0.9994 Absorbance, λ = 254 nm Analyte concentration [mM] OH NH2 F	 y = 0.6135x R² = 0.9997 Analyte [mM] Abs (analyte) / Abs (standard)
11	 y = 242.77x R² = 0.9994 Absorbance, λ = 230 nm Analyte concentration [mM] OH NH2 Cl	 y = 2.2136x R² = 0.9978 Analyte [mM] Abs (analyte) / Abs (standard)
12	 y = 23.744x R² = 0.9995 Absorbance, λ = 280 nm Analyte concentration [mM] OH NH2 F3C	 y = 2.1553x R² = 0.9995 Analyte [mM] Abs (analyte) / Abs (standard)

13	 y = 24.989x R² = 0.9993 Absorbance, λ = 254 nm Analyte concentration [mM] OH NH2 CF3	 y = 0.56x R² = 0.9993 Analyte [mM] Abs (analyte) / Abs (standard)
14	 y = 167.32x R² = 0.9996 Absorbance, λ = 280 nm Analyte concentration [mM] OH NH2 O2N	 y = 0.3067x R² = 0.9996 Analyte [mM] Abs (analyte) / Abs (standard)
15	 y = 64.992x R² = 0.9908 Absorbance, λ = 280 nm Analyte concentration [mM] HO NH2 H3CO	 y = 0.7856x R² = 0.9898 Analyte [mM] Abs (analyte) / Abs (standard)
16	 y = 38.435x R² = 0.9985 Absorbance, λ = 280 nm Analyte concentration [mM] OH NH2 O	 y = 1.3341x R² = 0.9986 Analyte [mM] Abs (analyte) / Abs (standard)

V. Derivatization of Amino Alcohol Products



Commercially available product standards **4** – **16** were dissolved in DMSO to make 200 mM stock solutions. To derivatize the product standards, 4 - 20 µL of the 200 mM stock solution was added to 310 µLof KPi (0.1 M, pH 8.0) containing 150 mM sodium chloride to mimic enzymatic reaction in whole cells. Saturated sodium bicarbonate solution (50 µL) and benzoyl chloride stock solution (10 µL, 10% v/v in acetonitrile) was added to the KPi solution. The mixture was allowed to shake at room temperature at 750 rpm for at least 3 hours.

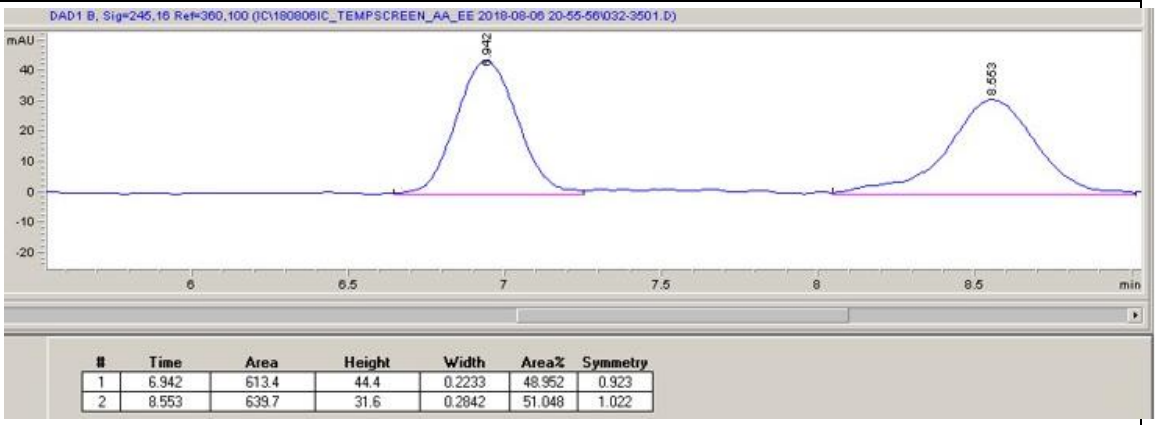
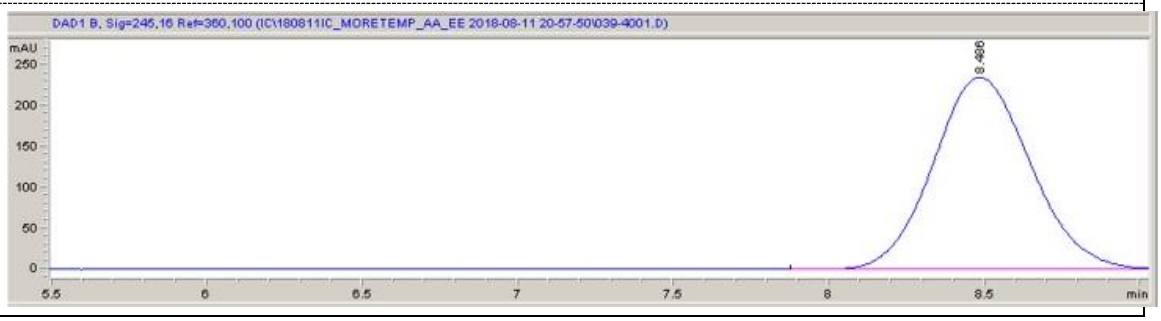
The supernatant of centrifuged whole cell reaction mixtures quenched with acetonitrile was dried overnight in the fume hood prior to derivatization, to remove acetonitrile. Once the volume of the supernatant was roughly under 300 µL, saturated sodium bicarbonate solution (50 µL) and benzoyl chloride stock solution (10 µL, 10% v/v in acetonitrile) was added. Greater than 10-fold excess of benzoyl chloride was used for all derivatization. The mixture was vortexed and then allowed to shake at room temperature at 750 rpm for at least 5 hours. Products after derivatization were extracted with ethyl acetate, dried, and re-dissolved in 40% hexanes in ethyl acetate for the determination of enantiomeric excess. Purified compounds were dissolved in 40% isopropanol (IPA) in hexanes.

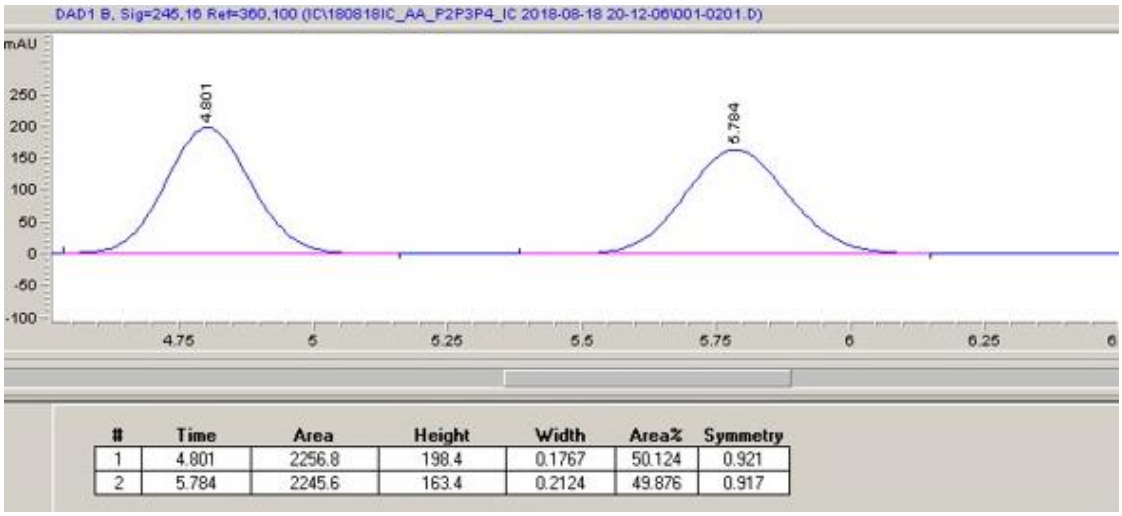
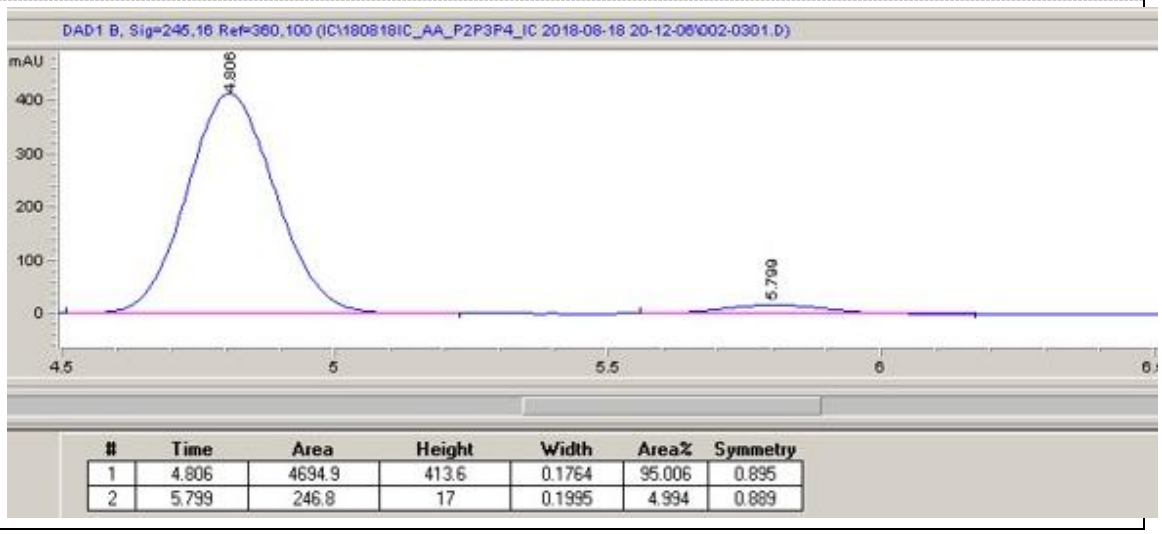
VI. Determination of Enantioselectivity and Assignment of Absolute Stereochemistry

Absolute stereochemistry was assigned using commercial standards of (*S*)-2-amino-1-phenylethanol and (*R*)-2-amino-1-(4-methoxyphenyl)ethanol along with their racemic standards, **9** and **4**. Comparison to the enzymatic reactions reveals the enzymatic major products to be the (*R*)-enantiomer. Absolute stereochemistry of other aminoalcohol products was assigned by analogy to be the (*R*)-enantiomer. Samples were analyzed using normal phase chiral HPLC using hexanes (hex) and isopropanol (IPA) as the mobile phase. All chiral HPLC traces were taken after derivatization with benzoyl chloride. All spectra were taken at 245 nm. For the analysis of products **4** – **15**, Chiralpak IC column was used. Chiralcel OJ-H column was used for the analysis of product **16**.

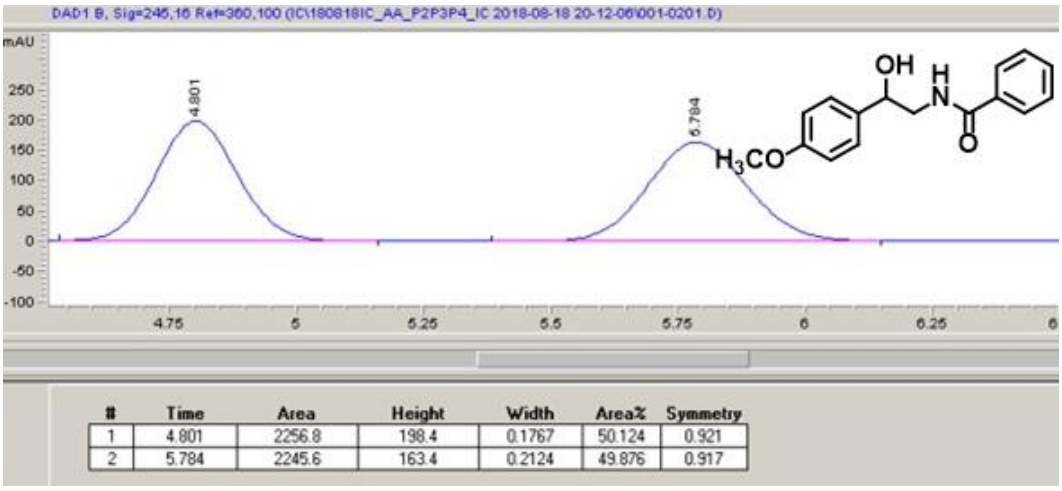
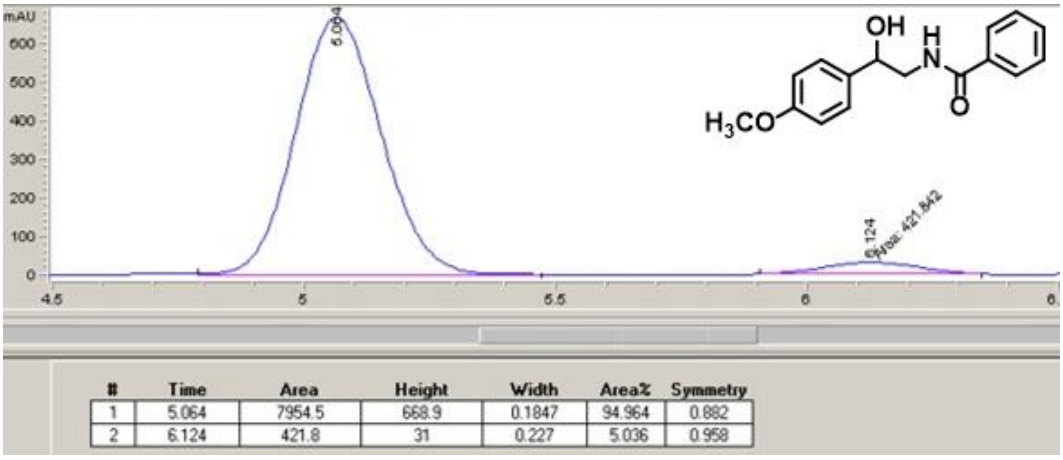
Additionally, the commercial standards of (*S*)-2-amino-1-phenylethanol and (*R*)-2-amino-1-(4-methoxyphenyl)ethanol were dissolved in the supernatant of centrifuged whole cell reaction mixtures quenched with acetonitrile, and were dried overnight in the fume hood. The standards were then derivatized by adding saturated sodium bicarbonate solution (50 µL) and benzoyl chloride stock solution (10 µL, 10% v/v in acetonitrile). Inversion or decrease in ee was not observed in this experiment.

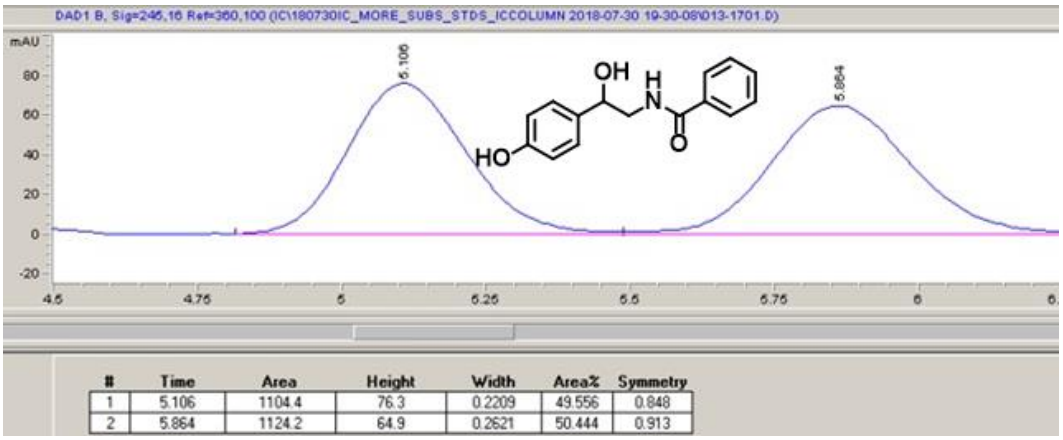
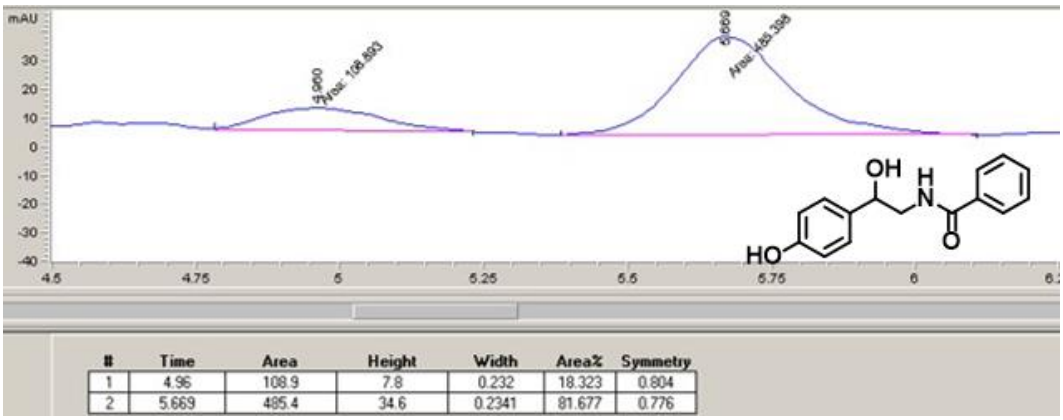
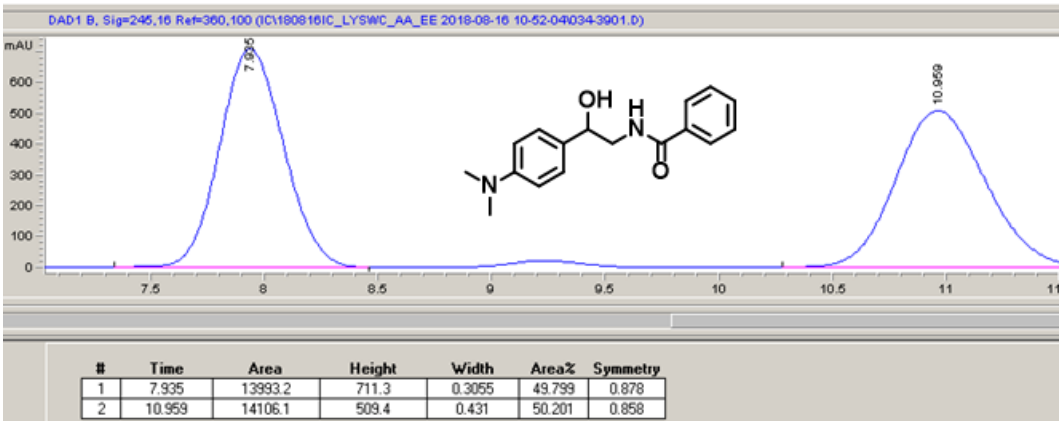
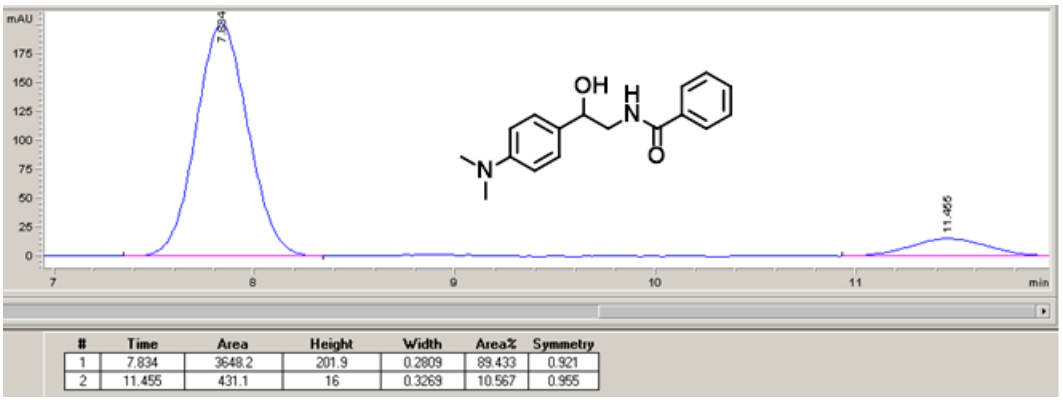
Assignment of absolute stereochemistry.

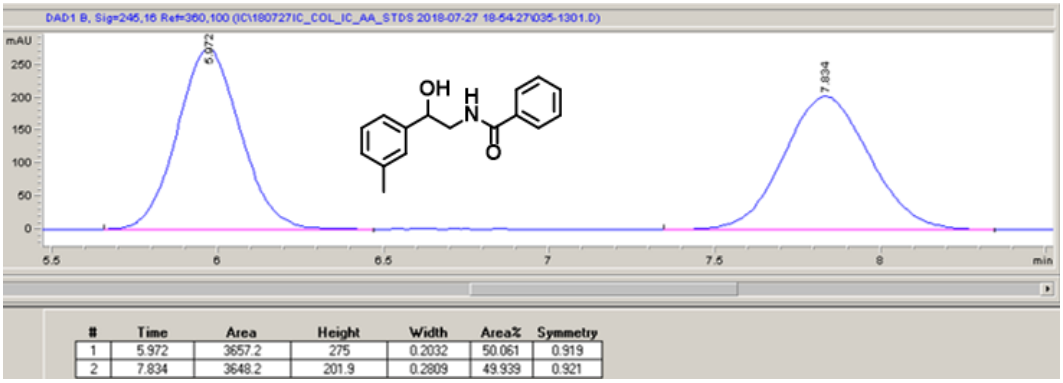
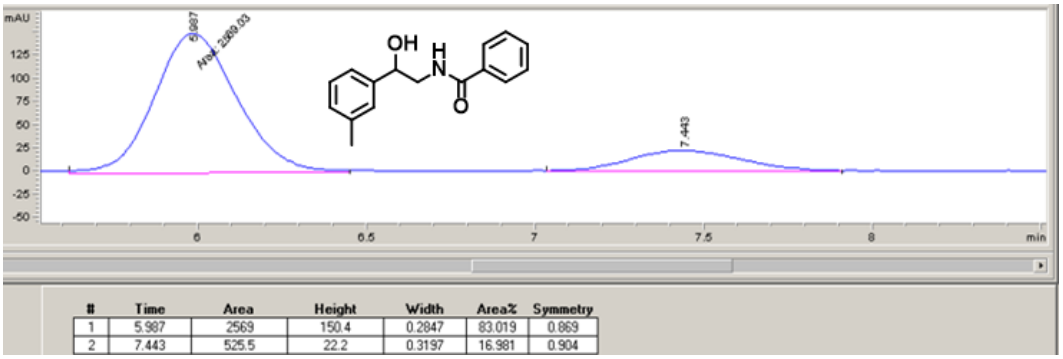
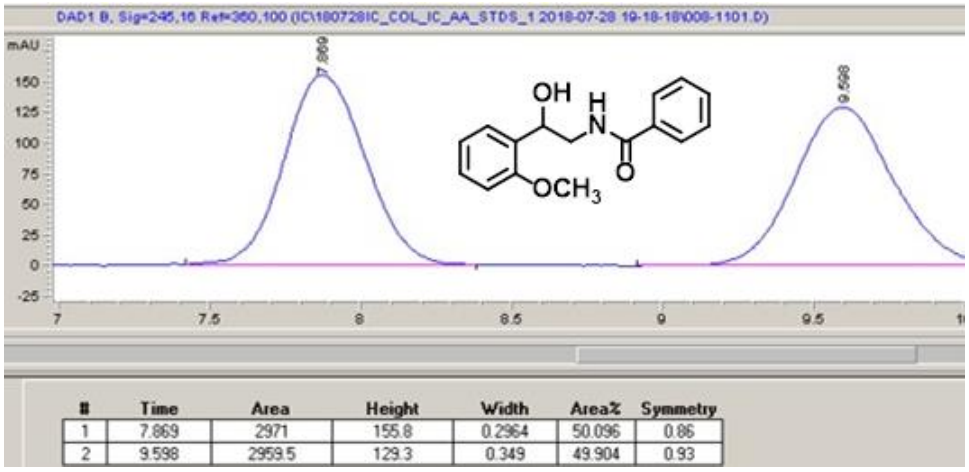
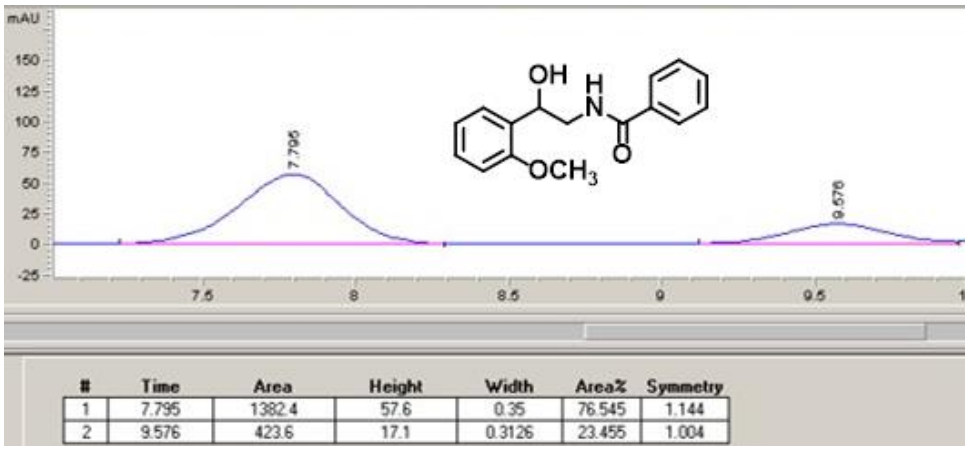
Standard / Method	HPLC trace
Derivatized 9(racemic) 85% hex. 15% IPA 2 mL/min 12 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 6.942 | 613.4 | 44.4 | 0.2233 | 48.952 | 0.923 | | 2 | 8.553 | 639.7 | 31.6 | 0.2842 | 51.048 | 1.022 |
Derivatized 9 (*S*) enantiomer standard	

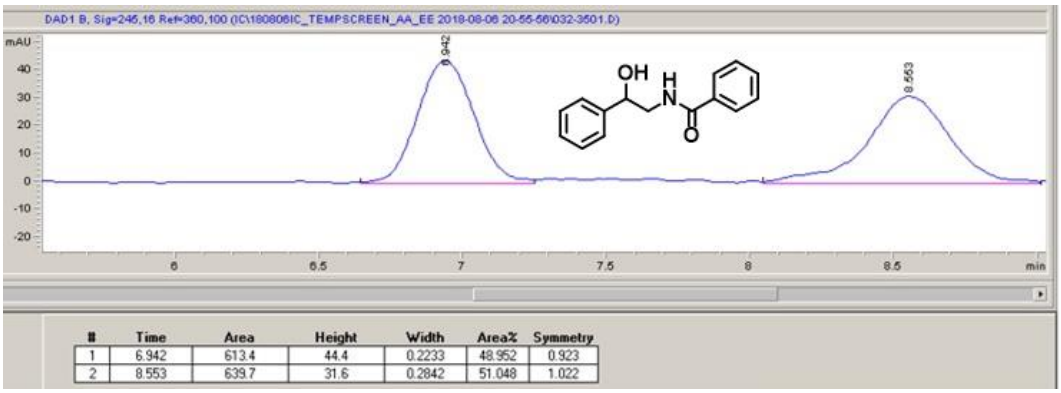
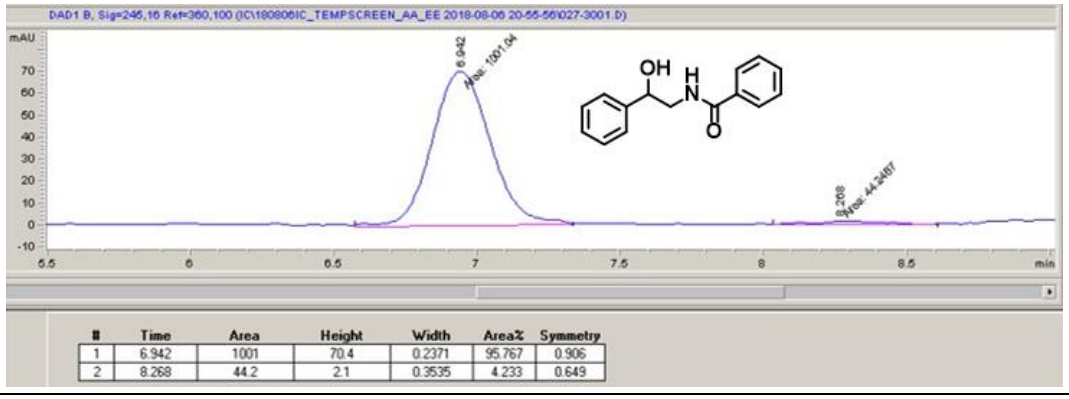
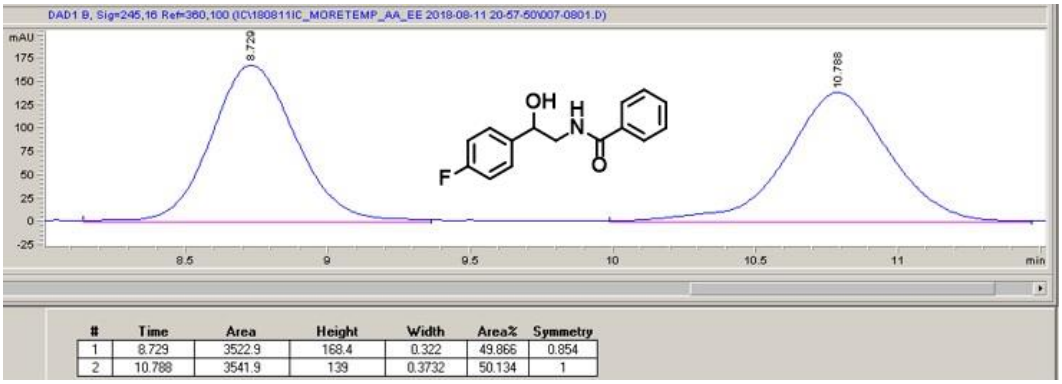
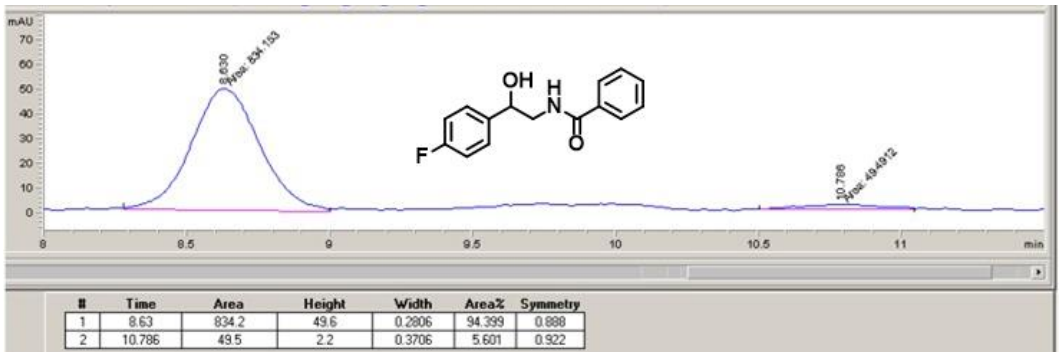
Derivatized 4 (racemic) 80% hex. 20% IPA 2.5 mL/min 10 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 4.801 | 2256.8 | 198.4 | 0.1767 | 50.124 | 0.921 | | 2 | 5.784 | 2245.6 | 163.4 | 0.2124 | 49.876 | 0.917 |
Derivatized 4 (*R*) enantiomer standard	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 4.806 | 4694.9 | 413.6 | 0.1764 | 95.006 | 0.895 | | 2 | 5.799 | 246.8 | 17 | 0.1995 | 4.994 | 0.889 |

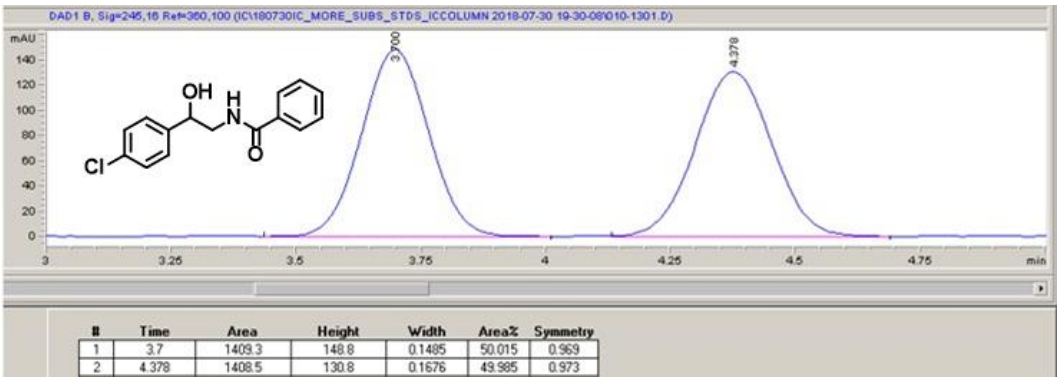
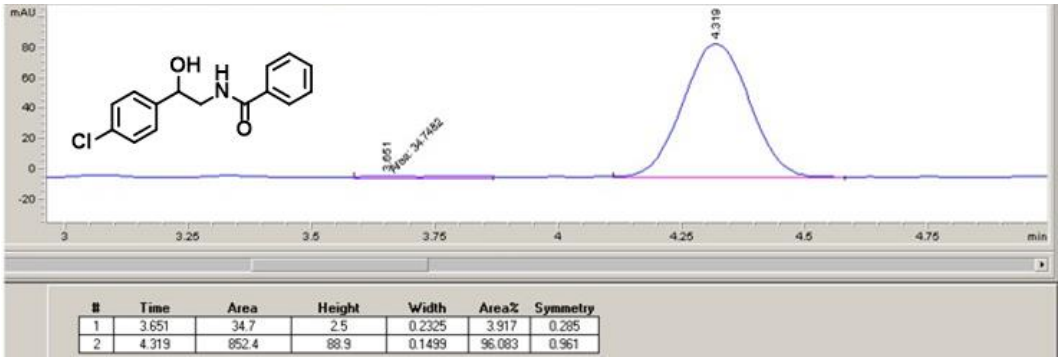
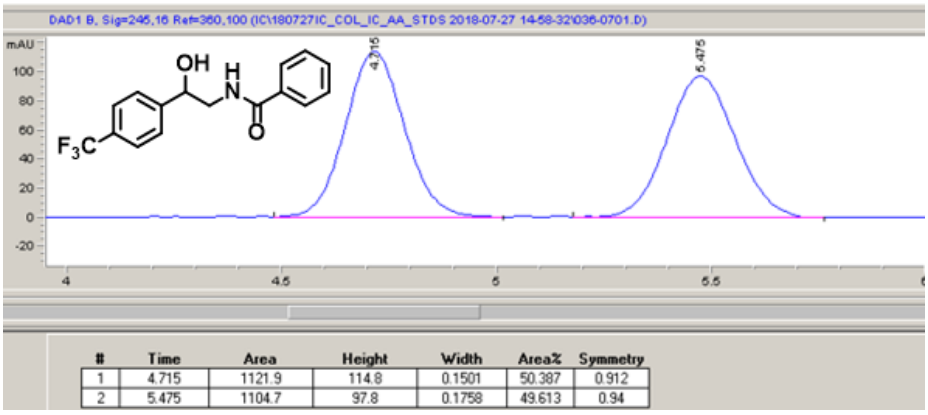
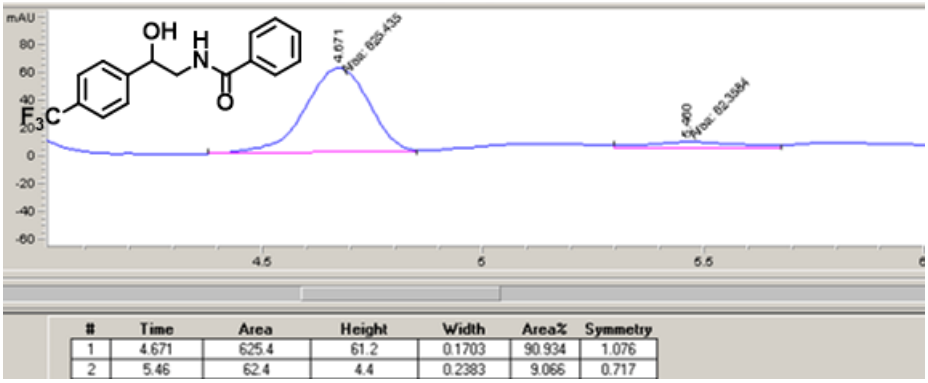
Determination of enantiomeric excess for P1 – P11.

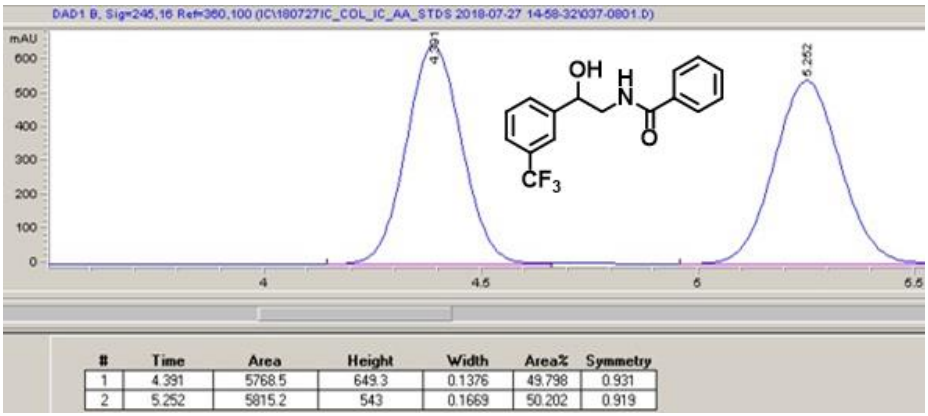
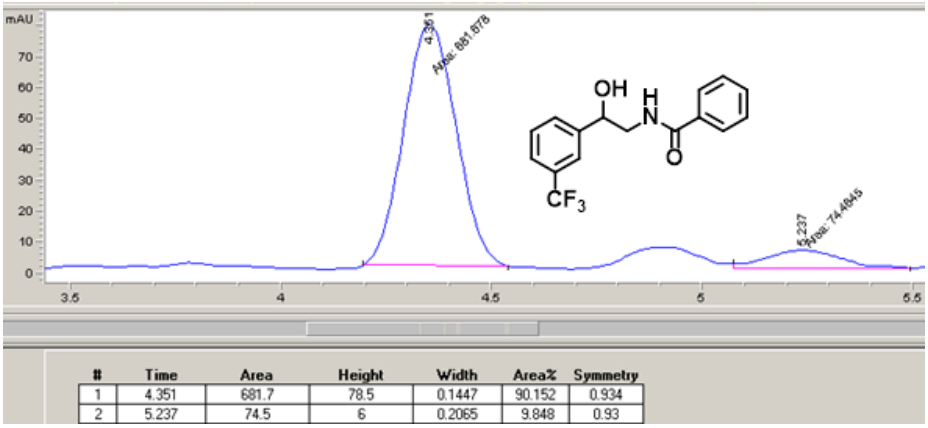
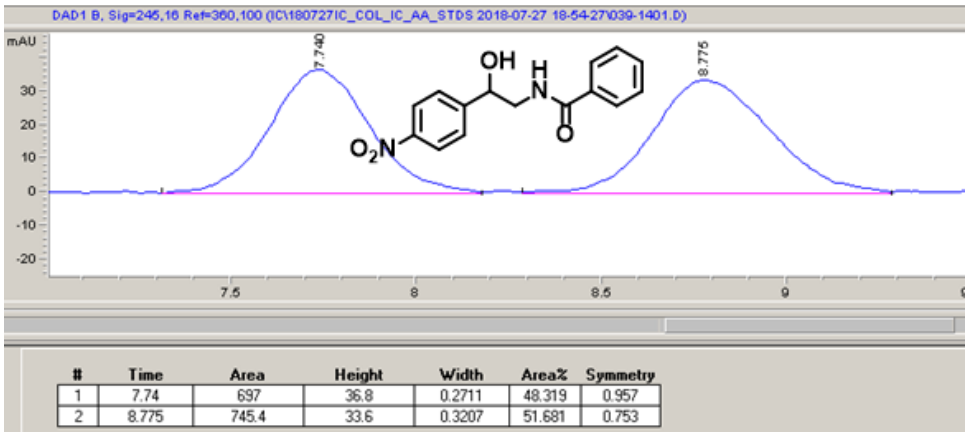
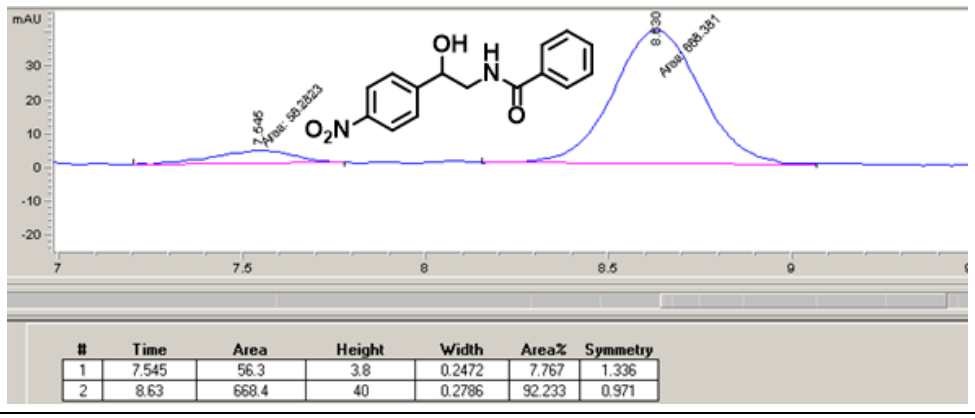
Analyte	Chiral HPLC trace
Derivatized 4 Racemic standard	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 4.801 | 2256.8 | 198.4 | 0.1767 | 50.124 | 0.921 | | 2 | 5.784 | 2245.6 | 163.4 | 0.2124 | 49.876 | 0.917 |
Derivatized 4 Enzymatic reaction **(90% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 5.064 | 7954.5 | 668.9 | 0.1847 | 94.964 | 0.882 | | 2 | 6.124 | 421.8 | 31 | 0.227 | 5.036 | 0.958 |

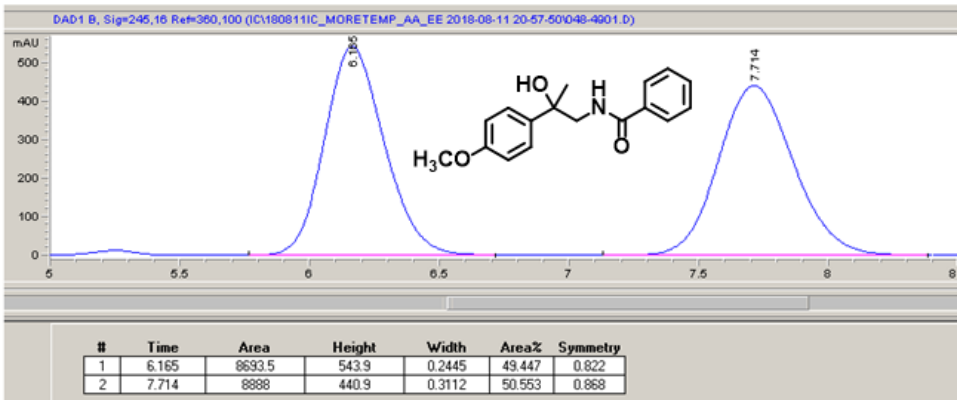
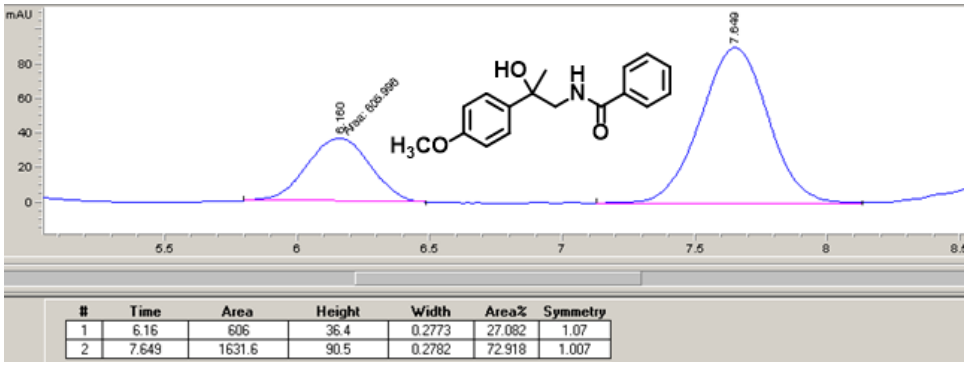
Derivatized 5 Racemic standard 80% hex. 20% IPA 2.5 mL/min 12 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 5.106 | 1104.4 | 76.3 | 0.2209 | 49.556 | 0.848 | | 2 | 5.864 | 1124.2 | 64.9 | 0.2621 | 50.444 | 0.913 |
Derivatized 5 Enzymatic reaction **(63% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 4.96 | 108.9 | 7.8 | 0.232 | 18.323 | 0.804 | | 2 | 5.669 | 485.4 | 34.6 | 0.2341 | 81.677 | 0.776 |
Derivatized 6 Racemic standard 80% hex. 20% IPA 2.5 mL/min 12 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 7.935 | 13993.2 | 711.3 | 0.3055 | 49.799 | 0.878 | | 2 | 10.959 | 14106.1 | 509.4 | 0.431 | 50.201 | 0.858 |
Derivatized 6 Enzymatic reaction **(80% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 7.834 | 3648.2 | 201.9 | 0.2809 | 89.433 | 0.921 | | 2 | 11.455 | 431.1 | 16 | 0.3269 | 10.567 | 0.955 |

Derivatized 7 Racemic standard 85% hex. 15% IPA 2.5 mL/min 10 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 5.972 | 3657.2 | 275 | 0.2032 | 50.061 | 0.919 | | 2 | 7.834 | 3648.2 | 201.9 | 0.2809 | 49.939 | 0.921 |
Derivatized 7 Enzymatic reaction **(67% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 5.987 | 2569 | 150.4 | 0.2847 | 83.019 | 0.869 | | 2 | 7.443 | 525.5 | 22.2 | 0.3197 | 16.981 | 0.904 |
Derivatized 8 Racemic standard 80% hex. 20% IPA 2.5 mL/min 12 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 7.869 | 2971 | 155.8 | 0.2964 | 50.096 | 0.86 | | 2 | 9.598 | 2959.5 | 129.3 | 0.349 | 49.904 | 0.93 |
Derivatized 8 Enzymatic reaction **(53% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 7.795 | 1382.4 | 57.6 | 0.35 | 76.545 | 1.144 | | 2 | 9.576 | 423.6 | 17.1 | 0.3126 | 23.455 | 1.004 |

Derivatized 9 Racemic standard 85% hex. 15% IPA 2 mL/min 12 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 6.942 | 613.4 | 44.4 | 0.2233 | 48.952 | 0.923 | | 2 | 8.553 | 639.7 | 31.6 | 0.2842 | 51.048 | 1.022 |
Derivatized 9 Enzymatic reaction **(92% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 6.942 | 1001 | 70.4 | 0.2371 | 95.767 | 0.906 | | 2 | 8.268 | 44.2 | 2.1 | 0.3535 | 4.233 | 0.649 |
Derivatized 10 Racemic standard 90% hex. 10% IPA 2 mL/min 12 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 8.729 | 3522.9 | 168.4 | 0.322 | 49.866 | 0.854 | | 2 | 10.788 | 3541.9 | 139 | 0.3732 | 50.134 | 1 |
Derivatized 10 Enzymatic reaction **(88% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 8.63 | 834.2 | 49.6 | 0.2806 | 94.399 | 0.888 | | 2 | 10.786 | 49.5 | 2.2 | 0.3706 | 5.601 | 0.922 |

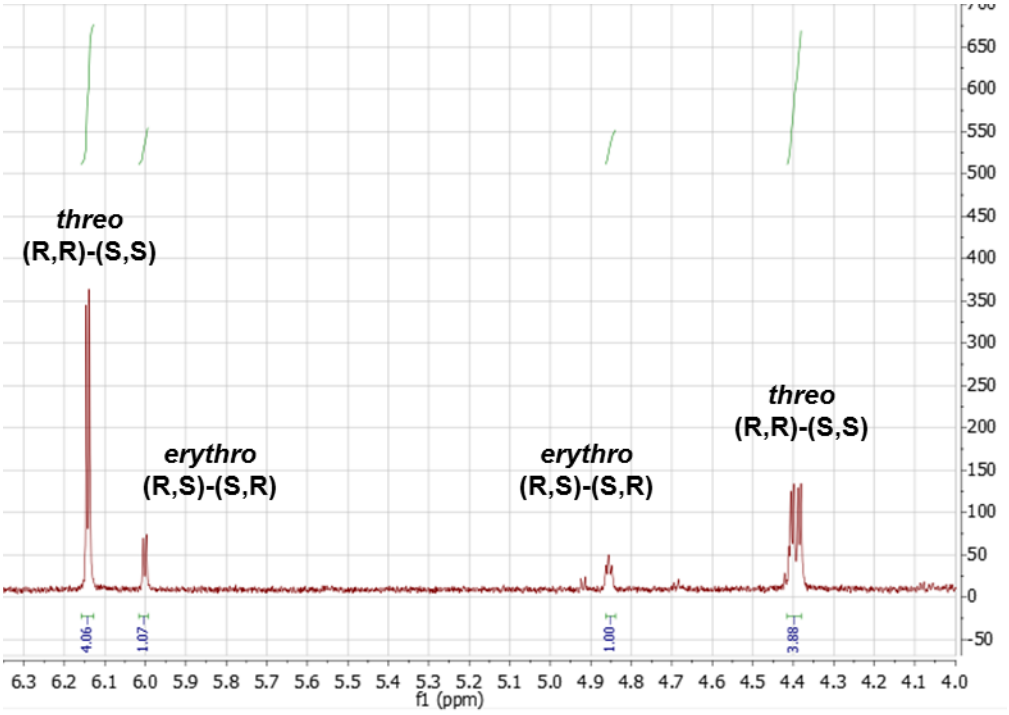
Derivatized 11 Racemic standard 90% hex. 10% IPA 2 mL/min 12 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 3.7 | 1409.3 | 148.8 | 0.1485 | 50.015 | 0.969 | | 2 | 4.378 | 1408.5 | 130.8 | 0.1676 | 49.985 | 0.973 |
Derivatized 11 Enzymatic reaction **(92% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 3.651 | 34.7 | 2.5 | 0.2325 | 3.917 | 0.285 | | 2 | 4.319 | 852.4 | 88.9 | 0.1499 | 96.083 | 0.961 |
Derivatized 12 Racemic standard 90% hex. 10% IPA 2 mL/min 12 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 4.715 | 1121.9 | 114.8 | 0.1501 | 50.387 | 0.912 | | 2 | 5.475 | 1104.7 | 97.8 | 0.1758 | 49.613 | 0.94 |
Derivatized 12 Enzymatic reaction **(82% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 4.671 | 625.4 | 61.2 | 0.1703 | 90.934 | 1.076 | | 2 | 5.46 | 62.4 | 4.4 | 0.2383 | 9.066 | 0.717 |

Derivatized 13 Racemic standard 90% hex. 10% IPA 2 mL/min 10 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 4.391 | 5768.5 | 649.3 | 0.1376 | 49.798 | 0.931 | | 2 | 5.252 | 5815.2 | 543 | 0.1669 | 50.202 | 0.919 |
Derivatized 13 Enzymatic reaction **(80% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 4.351 | 681.7 | 78.5 | 0.1447 | 90.152 | 0.934 | | 2 | 5.237 | 74.5 | 6 | 0.2065 | 9.848 | 0.93 |
Derivatized 14 Racemic standard 85% hex. 15% IPA 2.5 mL/min 12 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 7.74 | 697 | 36.8 | 0.2711 | 48.319 | 0.957 | | 2 | 8.775 | 745.4 | 33.6 | 0.3207 | 51.681 | 0.753 |
Derivatized 14 Enzymatic reaction **(85% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 7.545 | 56.3 | 3.8 | 0.2472 | 7.767 | 1.336 | | 2 | 8.63 | 668.4 | 40 | 0.2786 | 92.233 | 0.971 |

Derivatized 15 Racemic standard 80% hex. 20% IPA 2.5 mL/min 10 minutes	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 6.165 | 8693.5 | 543.9 | 0.2445 | 49.447 | 0.822 | | 2 | 7.714 | 8888 | 440.9 | 0.3112 | 50.553 | 0.868 |
Derivatized 15 Enzymatic reaction **(45% ee)**	 | # | Time | Area | Height | Width | Area% | Symmetry | |---|---|---|---|---|---|---| | 1 | 6.16 | 606 | 36.4 | 0.2773 | 27.082 | 1.07 | | 2 | 7.649 | 1631.6 | 90.5 | 0.2782 | 72.918 | 1.007 |

Diastereomer assignment and ee determination of enzymatic reaction with *trans*-anethole .

The ^{1}H-NMR spectrum of commercially available racemic standard 2-amino-1-(4-methoxyphenyl)propan-1-ol (**16**) was taken, and the diastereomeric ratio of this racemic standard was determined to be 4:1 between (1*R*,2*R*)-(1*S*,2*S*)-2-amino-1-(4-methoxyphenyl)propan-1-ol and (1*R*,2*S*)-(1*S*,2*R*)-2-amino-1-(4-methoxyphenyl)propan-1-ol. Full spectrum of product **15** was: ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.25 (s, 3H), 7.32 (d, *J* = 8.6 Hz, 2H), 6.98 (d, *J* = 8.5 Hz, 2H), 6.15 (d, *J* = 3.7 Hz, 1H, from *threo* diastereomer), 5.99 (d, *J* = 4.1 Hz, 1H, from *erythro* diastereomer), 4.85 (m, 1H, from *erythro* diastereomer), 4.36 (dd, *J* = 9, 3.5 Hz, 1H, from *threo* diastereomer), 3.82 (s, 3H), 3.26 (s, 1H), 0.98 (d, *J* = 6.5 Hz, 3H). The diastereomers were assigned by comparison to the ^{1}H-NMR spectra of both diastereomers from the literature.[12 13 14]

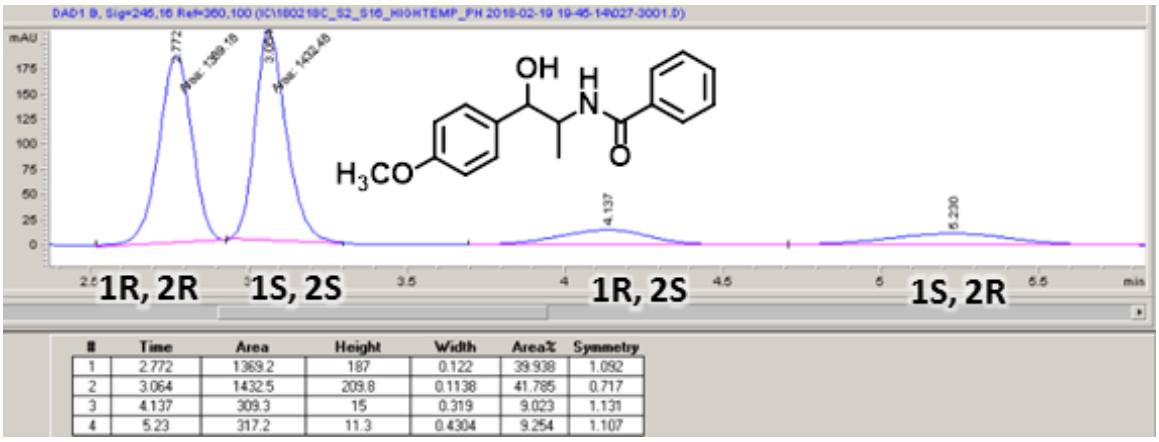


Next, chiral HPLC trace of commercial standard **16** after benzoyl chloride derivatization was taken. Two pairs of peaks with the absorbance ratio of 4:1 were identified, and were assigned as (1*R*,2*R*)-(1*S*,2*S*)-**16** and (1*R*,2*S*)-(1*S*,2*R*)-**16**, respectively. Finally, chiral HPLC trace of **16** obtained by enzymatic reaction was obtained after benzoyl chloride derivatization. The absolute configuration of the major diastereomer was assigned by analogy.

Derivatized 16

Racemic standard

85% hex.
15% IPA
2 mL/min
12 minutes

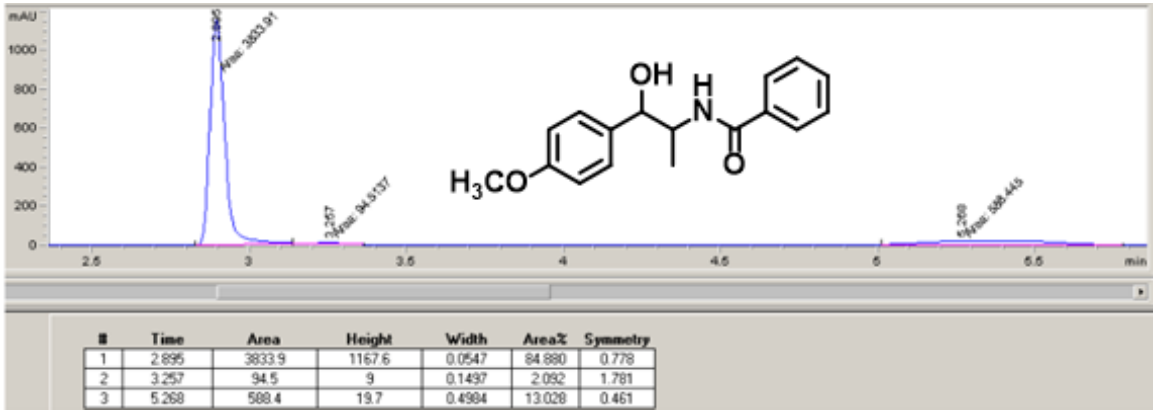


#	Time	Area	Height	Width	Area%	Symmetry
1	2.772	1369.2	187	0.122	39.938	1.092
2	3.064	1432.5	209.8	0.1138	41.785	0.717
3	4.137	309.3	15	0.319	9.023	1.131
4	5.23	317.2	11.3	0.4304	9.254	1.107

Derivatized 16

Enzymatic reaction

(87:13 dr)
(95% ee)



#	Time	Area	Height	Width	Area%	Symmetry
1	2.895	3833.9	1167.6	0.0547	84.880	0.778
2	3.257	94.5	9	0.1497	2.092	1.781
3	5.268	588.4	19.7	0.4984	13.028	0.461

VII. Preparative Scale Biocatalytic Reactions

BL21 E. cloni cells co-transformed with the pEC86 plasmid encoding ccmABCDEFG and pET22b(+) plasmid encoding *Rma* TQL variant were grown overnight in 15 mL $LB_{amp/chl}$ (37 °C, 230 rpm). $HB_{amp/chl}$ medium (800 mL) in a 2,000 mL flask was inoculated with 10 mL of the overnight culture. The HB culture was shaken at 37 °C and 250 rpm for 1 hours and 30 minutes, or until OD_{600} was 0.4-0.6. The culture was cooled on ice bath for 40 minutes, and then induced with 20 μM IPTG and 200 μM 5-aminolevulinic acid (final concentrations). The culture was allowed to shake for 22 hours at 200 rpm in an incubator with temperature reduced to 22.5 °C. Cells expressing the TQL variant were pelleted by centrifugation (4,500 g, 7 minutes, 4 °C), resuspended in 0.1 M potassium phosphate buffer (pH 8.0) containing 150 mM sodium chloride, and adjusted to OD_{600} = 60. The cell suspension was then poured into a 125 mL Erlenmeyer flask, sealed with a rubber cap and parafilm, put on ice bath, and degassed by sparging with argon for 30 minutes. The vial was then transferred into an anaerobic chamber. 80 mL of argon-purged cells were transferred into a new 125 mL Erlenmeyer flask, and an oxygen depletion system (5 mL, 14,000 U/mL catalase and 1,000 U/mL glucose oxidase in 0.1 M KPi, pH 8.0) and glucose solution (10 mL, 250 mM in 0.1 M KPi, pH 8.0) were subsequently added. Finally, nitrene precursor **N1** and substrate **S1** (5 mL each, 400 mM in DMSO) were added. The final reaction volume was 100 mL, and the amount of substrates added was 2.0 mmol each. The flask was sealed and the reaction proceeded at room temperature for 24 hours with stirring. The leftover cell suspension was used to determine the protein concentration using hemochrome assay.

After the reaction was frozen overnight and thawed at room temperature, 200 mL of acetonitrile was added, and the mixture was shaken at room temperature for an hour. The mixture was transferred to 50 mL falcon tubes and then centrifuged (5,000 g, 10 minutes). The analytical yield was determined by analyzing 200 μL of the supernatant with LC-MS (XDB-C8 column). The 200 μL sample was then dried, derivatized, and extracted into hexanes and ethyl acetate mixture to determine the analytical ee. The remaining supernatant was concentrated to less than 10 mL, filtered, and purified by silica gel column chromatography or semi-preparative reverse-phase HPLC column (XDB-C18 column).

a. 2-Amino-1-(4-methoxyphenyl)ethanol (product 4).

The reaction was performed on 2.0-mmol scale as described. The product was purified by C18 chromatography (5 to 100% acetonitrile/water). Analytical yield of 80% and ee of 88% was measured. After purification and evaporation of solvent under reduced pressure, 204 mg (61% yield) of the product was obtained with 85% ee. This compound is known in the literature.[15]

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.34 (br s, 2H), 7.42 (d, *J* = 8.6 Hz, 2H), 7.03 (d, *J* = 8.5 Hz, 2H), 4.92 (m, 1H), 3.81 (s, 3H), 3.15 (m, 2H).

^{13}C NMR (400 MHz, DMSO-d_6) δ 158.8 (1C), 135.7 (1C), 127.5 (2C), 115.8 (2C), 74.5 (1C), 55.4 (1C), 50.5 (1C).

b. 2-Amino-1-(4-methoxyphenyl)propan-1-ol (product 16).

Two reactions on 0.5-mmol scale with the final reaction volume of 24 mL in 50 mL Erlenmeyer flasks were performed using whole cells resuspended to OD_{600} = 60. After the analytical yield (84%), dr (87:13), and ee (92%) were determined, the supernatants of the acetonitrile-quenched and centrifuged reaction mixture were combined and concentrated to less than 1 mL. The product was purified using semi-preparative HPLC (XDB-C18 column, 5% to 95% acetonitrile/water with 0.1% acetic acid). The fractions containing the product were combined; the solvent was evaporated under reduced pressure. The product was obtained in 52% yield (95 mg) and the major diastereomer (dr 90:10) *threo*-2-amino-1-(4-methoxyphenyl)propan-1-ol was obtained with 90% ee.

Characterization for *threo*-**16**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.95 (br s, 2H), 7.27 (d, *J* = 8.8 Hz, 2H), 6.95 (d, *J* = 8.6 Hz, 2H), 6.01 (s, *J* = 3.6 Hz, 1H), 4.38 (d, *J* = 8.8 Hz, 1H), 3.74 (s, 3H), 3.17 (s, 1H), 0.95 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (400 MHz, DMSO-d_6) δ 159.4 (1C), 133.8 (1C), 125.1 (2C), 114.1 (2C), 74.5 (1C), 53.6 (1C), 52.8 (1C), 15.5 (1C).

VIII. Mechanistic Studies

a. Aminohydroxylation in $H_2{}^{18}O$ using purified protein.

To each of three 2-mL screw cap vials, 200 μL of isotope-labeled water ($H_2{}^{18}O$) and 160 μL of 0.1 M KPi buffer (pH 8.0, with 150 mM NaCl) were added. Purified TQL variant was subsequently added to the final protein concentration of 20 μM in the total reaction volume of 400 μL. Sodium dithionite solution (15 μL/vial, 10 mg/mL in the 0.1 M KPi buffer) was added to the vials, and the vials were moved into anaerobic chamber. Nitrene source **1** and substrate **3** (10 μL each, 400 mM stocks in DMSO) were added separately. The vials were capped and allowed to shake at room temperature at 700 rpm. After 24 hours, the vials were taken out of the anaerobic chamber, and the reaction was quenched by adding acetonitrile (800 μL) to each of the vials. The reaction mixtures were transferred to 1.7-mL Eppendorf tubes, vortexed, and then centrifuged (14,000 g, 5 minutes) to separate the coagulated protein from supernatant. The supernatant from each tube was then transferred to a 2-mL screw cap vial, and was analyzed using LC-MS (5% - 95% water to acetonitrile with 0.1% acetic acid). Based on the mass peak areas of unlabeled product **4** ($M+H^+$ = 168) and ^{18}O-labeled product **4** ($M+H^+$ = 170), 47% of product **4** was ^{18}O-labeled when 50% of the reaction buffer was of $H_2{}^{18}O$.

b. Synthesis of racemic 2-phenylaziridine and separation of enantiomers.

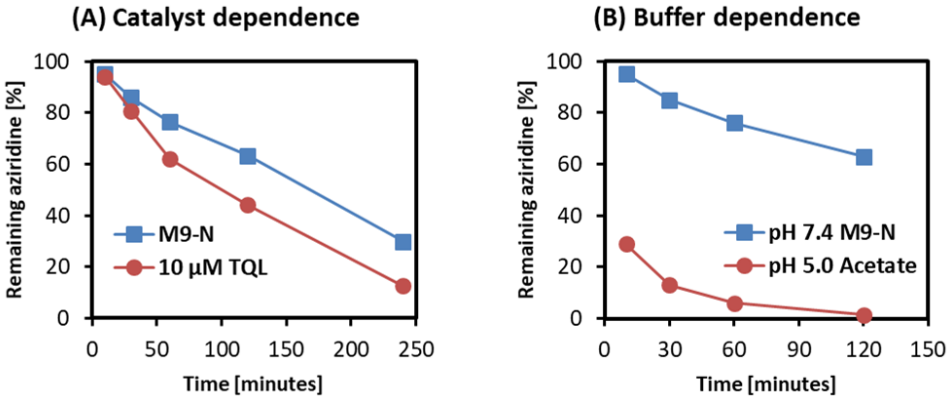
N-(p-tolylsulfonyl)-2-phenylaziridine was synthesized as previously reported in the procedure of T. Ando and coworkers.[16] To a degassed solution of acetonitrile (100 mL, sparged with argon for 20 minutes) was added chloramine-T trihydrate (4.23 g, 15.0 mmol) and I_2 (0.38 g, 1.5 mmol, 10 mol%). The headspace was then evacuated and filled again with argon three times. Next, styrene (3.12 g, 30.0 mmol) was added and the reaction was stirred at room temperature for 18 hours. The reaction mixture was diluted into 100 mL dichloromethane. After adding 50 mL water, the mixture was extracted two times with dichloromethane (100 mL each). The combined organic solvent was concentrated under reduced pressure. Purification by silica column chromatography (hexanes : ethyl acetate = 6 : 1, with 1% trimethylamine) afforded *N*-(p-tolylsulfonyl)-2-phenylaziridine (3.55 g, 13.0 mmol, 87% yield) as a white solid. Spectral data were consistent with literature.[17]

The tosyl-protected aziridine was then deprotected, following the procedure reported by R. A. Craig II and coworkers.[18] Magnesium turnings (0.50 g, 21.1 mmol) were activated by washing with HCl (5% in water). The magnesium turnings were resuspended in methanol (40 mL), and *N*-(p-tolylsulfonyl)-2-phenylaziridine (1.0 g, 3.7 mmol) in methanol (20 mL) was added to the suspension. The suspension was

sonicated at room temperature until complete consumption of the starting material as indicated by thin layer chromatography. Brine (200 mL) was then added to the suspension. The aqueous layer was extracted four times with dichloromethane (150 mL each), and the combined organic layer was concentrated under reduced pressure. Purification by silica column chromatography (ethyl acetate in hexanes, 25% to 60% gradient, with 1% trimethylamine) afforded 2-phenylaziridine (0.27 g, 2.3 mmol, 62% yield) as a clear oil. The spectral data were consistent with literature.[19] **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.38 – 7.28 (m, 2H), 7.28 – 7.16 (m, 3H), 3.02 (dd, J = 6.3, 3.4 Hz, 1H), 2.21 (d, J = 6.1 Hz, 1H), 1.79 (s, 1H), 0.74 (br s, 1H).

The racemic phenylaziridine product was dissolved in hexanes (200 mM). The two enantiomers were separated and purified by multiple injections with chiral HPLC using Chiracel OJ-H column (hexanes/isopropanol). The excess solvents were removed under reduced pressure to obtain (*S*)-(+)-2-phenylaziridine and (*R*)-(-)-2-phenylaziridine. The specific rotation of isolated (*R*)-(-)-2-phenylaziridine, $[\alpha]_D$ -30° (c 0.5, $CHCl_3$), was compared to the value reported in literature[18] to assign the absolute configuration. The specific rotation of the (S)-(+)-2-phenylaziridine was found to be $[\alpha]_D$ 29° (c 0.5, $CHCl_3$). Both enantiomers were immediately dissolved in DMSO (100 mM) and stored at -20 °C.

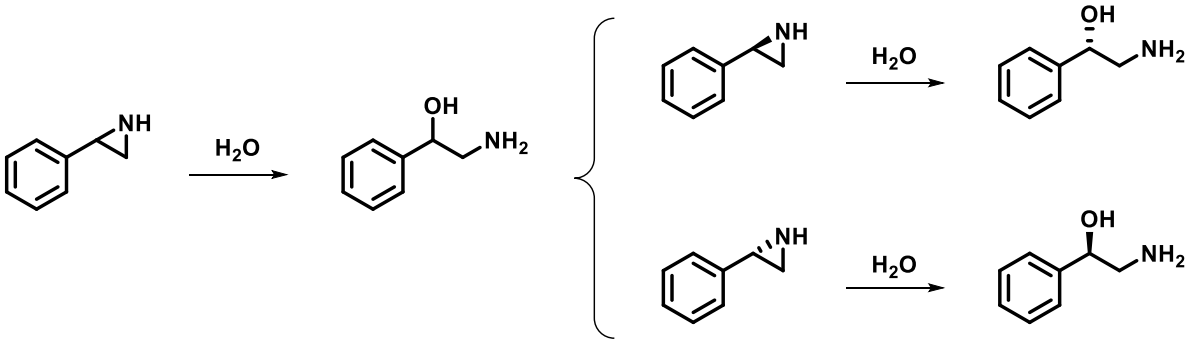
c. Hydrolysis time-course of 2-phenylaziridine.



To fifteen 1.7-mL Eppendorf tubes containing M9-N medium (300 µL/tube, pH 7.4), racemic phenylaziridine stock solution (10 µL/tube, 200 mM in DMSO) was added. The tubes were shaken at 700 rpm at room temperature for 4 hours. After 10 minutes, 30 minutes, 1 hours, 2 hours, and 4 hours, three Eppendorf tubes were taken out of the shaker. The solution in the tubes was basified by adding saturated sodium bicarbonate solution (50 µL/tube), and the remaining phenylaziridine was extracted with 50% ethyl acetate in hexanes. The amount of remaining phenylaziridine was determined by GC-MS analysis. This experiment was repeated using M9-N medium containing 10 µM TQL variant to monitor the

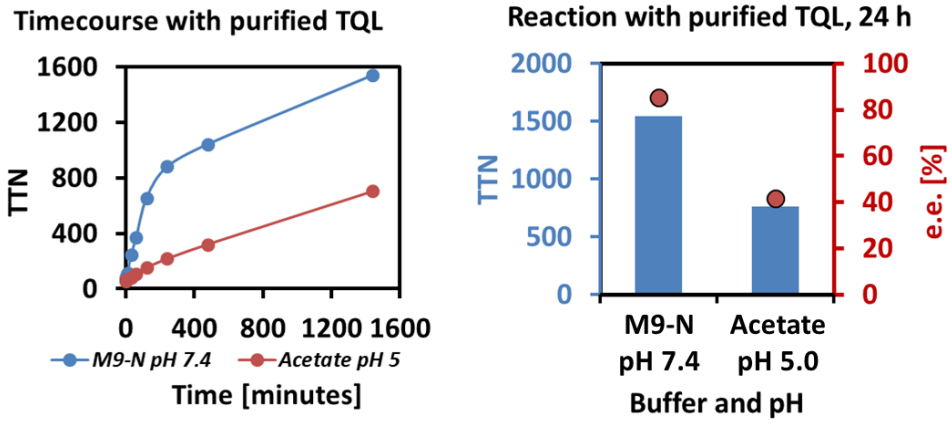
degradation of racemic 2-phenylaziridine in the presence of the protein catalyst. An additional degradation experiment was performed using sodium acetate buffer (pH 5.0) instead of M9-N medium were performed to study the effect of pH in aziridine hydrolysis. The hydrolysis was observed to be facilitated if purified TQL variant is present. The hydrolysis was much faster in acidic acetate buffer (pH 5.0) than in M9-N medium.

d. Stereospecificity of 2-phenylaziridine hydrolysis.



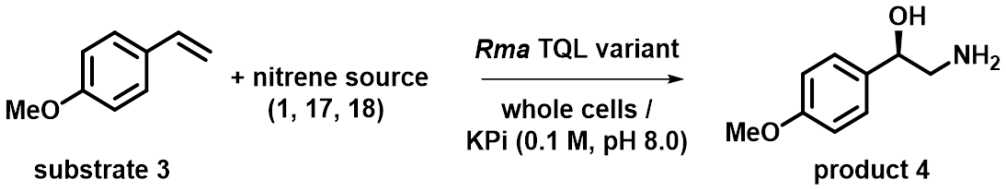
M9-N medium (300 µL/tube, pH 7.4) was added to six 1.7-mL Eppendorf tubes, and acetate buffer (300 µL/tube, pH 5.0) was added to another set of six 1.7-mL Eppendorf tubes. To three of the tubes containing M9-N medium and three of the tubes containing acetate buffer, purified TQL variant was added to the final concentration of 10 µM. Racemic 2-phenylaziridine (200 mM in DMSO), (*S*)-2-phenylaziridine, and (*R*)-2-phenylaziridine (100 mM in DMSO) were each added to two tubes with M9-N and two tubes with acetate buffer to the final concentration of 5 mM. All Eppendorf tubes were shaken at 700 rpm at room temperature for 6 hours. Saturated sodium bicarbonate solution (50 µL/tube) was added to the tubes, which were washed subsequently with 50% ethyl acetate in hexanes to remove any unhydrolyzed 2-phenylaziridine from 2-amino-1-phenylethanol, the hydrolysis product. Benzoyl chloride solution (10 µL/tube, 10% in acetonitrile) was added to the remaining aqueous layer, and the tubes were shaken at 700 rpm at room temperature for 4 hours. The derivatized 2-amino-1-phenylethanol was extracted with 50% ethyl acetate in hexanes, dried, and then dissolved again in hexanes. The stereochemistry of this hydrolysis product was determined by chiral HPLC using Chiralcel OJ-H column (hexanes/isopropanol). The retention time was compared to the retention times of racemic 2-amino-1-phenylethanol and (*S*)-2-amino-1-phenylethanol standards after derivatization. The (*R*)-2-phenylaziridine and (*S*)-2-phenylaziridine hydrolyzed to (*S*)-2-amino-1-phenylethanol and (*R*)-2-amino-1-phenylethanol, respectively, in both M9-N medium and acetate buffer with or without TQL variant. Racemic phenylaziridine hydrolyzed to racemic aminoalcohol.

e. Purified enzyme reactions in M9-N medium and acetate buffer.

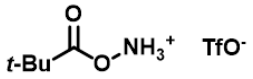
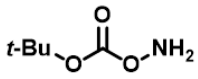
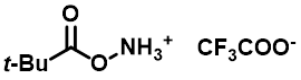


Four sets of time-course experiments with 11 time points were performed using purified TQL variant. Two sets were performed with M9-N medium (pH 7.4) as the solvent, whereas the other two sets were performed using acetate buffer (0.1 M, pH 5.0). Each set of experiments was conducted in the following manner. Reaction solvent (370 µL/vial) containing purified TQL variant (2 µM, final concentration) was pipetted to all eleven 2-mL screw cap vials. Sodium dithionite solution (10 µL/vial, 10 mg/mL in the matching reaction solvent) was added. The vials were moved into anaerobic chamber, where nitrene source **1** and substrate **3** (10 µL each, 200 mM stocks in DMSO) were added. The vials were capped and allowed to shake at room temperature at 700 rpm. After each time point (0.5, 1, 2, 5, 10, 30, 60, 120, 240, 480, and 1440 minutes) had passed, reaction in one vial was quenched by adding 800 µL acetonitrile. Vials containing quenched reactions were immediately taken out of the anaerobic chamber. The reaction mixtures were transferred to 1.7-mL Eppendorf tubes, vortexed, and centrifuged (14,000 g, 5 minutes) to separate the protein from supernatant. To determine the TTN, 200 µL of the supernatant was transferred to a screw cap GC vial with 0.4-mL vial insert, and was analyzed using LC-MS (5% - 95% water to acetonitrile with 0.1% acetic acid). The remaining supernatant was dried until acetonitrile was removed, and the product in the aqueous solvent was derivatized with benzoyl chloride to determine the ee.

f. **Aminohydroxylation of substrate 3 with other nitrene precursors**.



substrate 3 product 4

Nitrene source	Structure	TTN	e.e. after derivatization [%]
1		2300	90
17		1500	93
18		2100	92

We performed aminohydroxylation of **3** using three additional unprotected nitrene precursors in phosphate buffer (0.1 M, pH 8.0). Aminohydroxylation of **3** with precursor **17** yielded **4** with 93% ee, while **18** yielded **4** with 92% ee. It is noteworthy that the identity of the nitrene precursor and the presence of the acid salt can affect the selectivity even when the reaction intermediate is presumably identical.

Reactions were performed in whole cells expressing the TQL variant, resuspended in phosphate buffer (0.1 M, pH 8.0) to OD_{600} = 30, with 10 mM of each substrate. Reactions were set up inside an anaerobic chamber and were shaken at 600 rpm at room temperature for 18 hours. Results are the average of triplicate reactions. The values for e.e. were measured after derivatization with benzoyl chloride.

g. Aminohydroxylation of substrate 3 at higher temperatures using purified TQL variant.

Temperature	pH 7.4 M9-N medium		pH 5.0 acetate buffer	
	TTN	ee [%]	TTN	ee [%]
37 °C	2900	89	1700	57
42 °C	3200	77	2100	61
50 °C	3300	59	3000	55

Reactions were performed using purified TQL variant (10 μM final concentration) in the specified reaction buffer. Sodium dithionite solution (10 μL/reaction, 10 mg/mL in water) was added to reduce the iron cofactor of the hemoprotein. The final concentration of both reagents, **1** and **3**, was 5 mM. The total

reaction volume was 400 µL. Reactions were set up in an anaerobic chamber, capped, further sealed with parafilm, and then taken out of the anaerobic chamber. Reactions were shaken in an incubator adjusted to the specified reaction temperature at 300 rpm.

IX. Sequence Information

a. Gene sequence of the wildtype *Rma* cytochrome *c*.

gtttaactttaagaaggagatatacatATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCC
GGCGATGGCCATGACTGAAAGCGGGACTGCTGCACAAGACCCGGAAGCACTGGCAGCGGAAATTGGTCCGGTCA
AACAGGTGAGCCTGGGTGAACAGATTGATGCGGCCCTGGCGCAACAGGGAGAACAGCTCTTCAACACGTATTGT
ACTGCGTGCCACCGTCTGGATGAGCGTTTTATCGGACCGGCCCTGCGCGATGTTACCAAACGTCGTGGGCCGGTTT
ACATCATGAACGTGATGCTGAACCCGAATGGGATGATCCAGCGTCATCCGGTGATGAAACAGCTCGTGCAGGAAT
ATGGGACCATGATGACCGATATGGCCCTGAGTGAAGAACAAGCGCGCGCAATTCTGGAGTATCTGCGCCAGGTTG
CGGAAAACCAGCTCGAGCACCACCATCACCACCACTGA

[1:27]: (gray, lowercase) N-terminus homology region

[28:93]: (gray, uppercase) pelB

[94:468]: (black, uppercase) *Rma* cytochrome *c*

[469:495]: (gray, uppercase) *Xho*1, C-terminus His-Tag, stop codon

b. Gene sequence of *Rma* cytochrome *c* variant TQL.

gtttaactttaagaaggagatatacatATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCC
GGCGATGGCCATGACTGAAAGCGGGACTGCTGCACAAGACCCGGAAGCACTGGCAGCGGAAATTGGTCCGGTCA
AACAGGTGAGCCTGGGTGAACAGATTGATGCGGCCCTGGCGCAACAGGGAGAACAGCTCTTCAACACG**ACG**TGT
ACTGCGTGCCACCGTCTGGATGAGCGTTTTATCGGACCGGCCCTGCGCGATGTTACCAAACGTCGTGGGCCGGTTT
ACATCATGAACGTG**CA**GCTGAACCCGAATGGGATGATCCAGCGTCATCCGGTGATGAAACAGCTCGTGCAGGAAT
ATGGG**CTTG**TGA**GTCCG**GAT**GGT**GCCCTGAGTGAAGAACAAGCGCGCGCAATTCTGGAGTATCTGCGCCAGGTT
GCGGAAAACCAGctcgagcaccaccatcaccaccactga

Nucleotides marked in red are mutated from the wildtype gene.

c. Amino acid sequence of the wildtype *Rma* cytochrome *c*.

MKYLLPTAAAGLLLLAAQPAMAMTESGTAAQDPEALAAEIGPVKQVSLGEQIDAALAQQGEQLFNTYCTACHRLDERF
IGPALRDVTKRRGPVYIMNVMLNPNGMIQRHPVMKQLVQEYGTMMTDMALSEEQARAILEYLRQVAENQLEHHHH
HH*

[1:22]: (gray, uppercase) pelB

[23:147]: (black, uppercase) *Rma* cytochrome *c*

[148:157]: (gray, uppercase) Xho1, C-terminus His-Tag, stop codon

d. Amino acid sequence of variant TQL.

MKYLLPTAAAGLLLLAAQPAMAMTESGTAAQDPEALAAEIGPVKQVSLGEQIDAALAQQGEQLFNT**T**CTACHRLDERF
IGPALRDVTKRRGPVYIMNV**Q**LNPNGMIQRHPVMKQLVQEYG**LVSP**D**G**ALSEEQARAILEYLRQVAENQLEHHHHH
H*

Residues marked in red are mutated from the wildtype protein.

X. Supplementary References

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